BIOCHEMICAL GENETICS OF ORGANOPHOSPHATE RESISTANCE IN THE AUSTRALIAN SHEEP BLOWFLY, *LUCILIA CUPRINA*

by

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Declaration

The research carried out in the course of this investigation and the results presented in this thesis are, except where acknowledged, the original work of the author.

Kerrie-Ann Smyth

This thesis is dedicated to the "oldies"

Pop and Kitty Costin

and to the memory of

Aunty Ag, and

Nannie and Fred Smyth.

Thanks for your love, encouragement and support.

There's no harm in trying.
Nothing can harm you till it comes.
And it may never come.
Or if it comes it is something else again.
And those who say, "I'll try anything once",
often try nothing twice, three times,
arriving late at the gate of dreams worth dying for.

from Breathing Tokens by Carl Sandburg.

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2. Smyth, K. A., Russell, R. J. and Oakeshott, J. G. A cluster of at least three esterase genes in *Lucilia cuprina* includes malathion carboxylesterase and two other esterases implicated in resistance to organophosphates. *Biochem. Genet.* in press.

3. Smyth, K. A., Walker, V. K., Russell, R. J. and Oakeshott, J. G. Biochemical and physiological differences in the malathion carboxylesterase activities in malathion susceptible and resistant lines of the sheep blowfly, *Lucilia cuprina*. *Insect Biochem. Molec. Biol.* submitted.

4. Smyth, K. A., Boyce, T. M., Russell, R. J. and Oakeshott, J. G. Negative association between malathion and diazinon resistances in the Australian sheep blowfly, *Lucilia cuprina*. in preparation.

LIST OF ABBREVIATIONS

ACH	Acetylcholine
AChE	Acetylcholinesterase
ChA	Choline Acetyltransferase
DCNB	1,2-dichloro-4-nitrobenzene
DDT	Dichlorodiphenyl Trichloroethane
DEPA	Diethyl Phosphate
DETPA	Diethyl Phosphorothioate
DFP	Diisopropylfluorophosphate
DTT	Dithiothreitol
EDTA	Ethylene Dinitrilotetra-acetic Acid
EMS	Ethyl Methanesulphonate
GABA	γ-aminobutyric Acid
GSH	Glutathione
GST	Glutathione *S*-transferase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
MCE	Malathion Carboxylesterase
mfo	Mixed Function Oxidase
OTFP	3-octylthio-1,1,1-trifluoro-2-propanone
OP	Organophosphate
PAGE	Polyacrylamide Gel Electrophoresis
PBO	Piperonyl Butoxide
PTU	Phenylthiourea
TBTP	S,S,S-tributylphosphorotrithioate (DEF®)
TPP	Triphenylphosphate

SUMMARY

There are three discrete malathion carboxylesterase (MCE) phenotypes, low, intermediate and high in lines of *Lucilia cuprina* made homozygous for the fourth chromosome and in field-derived strains of this species. Malathion resistance is conferred by high MCE activity. Strains with high MCE activity are 1000-fold more resistant to malathion than those with either intermediate or low MCE activity. Intermediate and low MCE activity strains have similar adult LD_{50} values for malathion, although intermediate activity is 21-fold higher than low. In larvae of the high strain MCE activity is 1.5- and 4-fold greater than that in the intermediate and low MCE strains. The corresponding figures for adult high MCE activity are 1.6- and 33-fold greater than the intermediate and low activities, respectively. MCE activity is expressed throughout development and is primarily found in tissues involved in digestion and neurological function.

All three MCE phenotypes map to chromosome 4 and the gene(s) encoding their activities is closely linked to that conferring resistance to general organophosphate insecticides (OPs), *Rop1*. *Rop1* encodes another carboxylesterase gene esterase 3 (E3) and the non-staining phenotype of the E3 isozyme is correlated with resistance to general OPs. The genetics of MCE activity show that high and low MCE activity are allelic and are encoded by *Rmal*. The results for intermediate MCE activity show that it may be allelic to either *Rmal* or *Rop1*. Mapping of *Rmal*, *Rop1* and another esterase gene E9 reveal that there is a cluster of at least three esterase genes located on the left arm of chromosome 4 approximately four map units from the visible marker *bubbled wings*. Another esterase gene E4 maps outside the cluster four map units on the other side of *bubbled wings*.

The three MCE phenotypes can be divided according to their response to esterase inhibitors as well as their activities. Low adult MCE activity is distinguished by its relative insensitivity to the organophosphorus inhibitors paraoxon and triphenylphosphate (TPP) and is classified as a subclass II carboxylesterase. The low larval MCE activity is similar to that of the intermediate adult and larval activities with respect to inhibitor sensitivities. These three are inhibited by the lowest concentration of paraoxon, but not that of TPP and were classified as subclass I carboxylesterases. The larval and adult high MCE activities appear to be the same and although were also classified as subclass I carboxylesterases, they are distinct from the other two types of MCE activity because they are inhibited by all three concentrations of TPP. These data suggest that the MCE in the malathion resistant high line may be structurally different from the MCEs in the malathion susceptible intermediate and low lines.

A negative association between MCE and E3 activities was initially identified in strains used to map MCE and E3 activities. Those that showed the intermediate or high MCE phenotype were E3 staining and those that were MCE low were E3 non-staining. The negative association between MCE and E3 activities correlates directly with resistance to malathion or diazinon, a general OP. Flies resistant to malathion via the high MCE activity allele are susceptible to diazinon and conversely flies with low MCE and E3 non-staining activities are resistant to diazinon. Strains from the field screened for malathion resistance in the process of producing iso-chromosome 4 lines have similar MCE activities, sensitivities to TPP and paraoxon and malathion LD_{50} values as the original high MCE malathion resistant line, and the malathion resistance locus in a representative

of these screened lines maps to the same region of chromosome 4 as the esterase cluster. These data suggest that the MCE activities and malathion resistance in the screened lines are conferred by alleles at *Rmal*. All of these lines are E3 non-staining but four of the five tested are susceptible to diazinon. This is the first report of an E3 non-staining/diazinon susceptible phenotype.

All the MCE and E3 phenotypes observed in a sample of 100 individuals from each of five mass populations could be accounted for by the genotypes present in the iso-chromosome lines. Three important results were evident from the mass populations. The first was the presence of the E3 non-staining phenotype in a population collected before the use of OPs. The second was the generation of data that concur with the evidence from the screened iso-chromosome lines for a novel E3 phenotype, E3 non-staining/diazinon susceptible. Thirdly, the negative associations between MCE and E3 activities and malathion and diazinon resistances were also observed in the mass populations. Therefore, the negative association between malathion and diazinon resistance appears to occur in both field populations and in field derived iso-chromosome 4 lines.

The expectation that increased MCE activity is the result of either increased expression or a structural mutation affecting kinetics is not entirely fulfilled. In fact, subtle differences in kinetics may interact with changes in expression to confer malathion resistance. Moreover, the expression of diazinon resistance via E3 non-staining activity is significantly constrained by malathion resistance via high MCE activity. An extreme gametic disequilibrium exists between MCE and E3 activities that cannot be explained simply by the history of selection and must be the result of interactions of the mutant and native proteins encoded by the *Rop1* and *Rmal* loci.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The phenomenon of insecticide resistance is an excellent model system to study the adaptation of an organism in response to change in its environment. Our ability to control insect populations is seriously compromised by their ability to develop resistance to a wide range of insecticides. I am interested in the biochemical and genetic basis of evolutionary change, and insecticide resistance offers the rare opportunity to examine evolutionary change within an experimentally amenable time frame, where the selective agent is known, and to observe the effects of individual genes. I am using *Lucilia cuprina* (the Australian sheep blowfly) as a model to study the biochemical and genetic basis of insecticide resistance to organophosphates (OPs) in general and, more specifically, to malathion. A greater understanding of the biochemical and genetic basis of resistance may enable us to develop strategies to prevent or reverse insecticide resistance.

Insecticide resistance has typically evolved via the modification of one or a few traits under monogenic control (Georghiou, 1969; Roush and McKenzie, 1987; Roush and Daly, 1990). Resistance alleles are believed to be rare in field populations before exposure to insecticides (Crow, 1957). In the absence of insecticide, a resistance allele is thought to be at a selective disadvantage due to the biochemical and physiological perturbations the products of such an allele may generate, although such a disadvantage is not always easily evaluated (Georghiou and Taylor, 1986; Roush and McKenzie, 1987). However, under selection pressure imposed by insecticide application the resistance conferring allele will be favoured, resulting in greater fitness within the selective environment (Georghiou and Taylor, 1986). The rate at which the resistance allele spreads depends on several factors, including selection pressure, immigration of susceptible alleles into the population and the relative fitness of the resistance allele (Georghiou and Taylor, 1986; Roush and McKenzie, 1987).

The history of insecticide resistance has shown that many insect species are able to develop resistance to one or more classes of insecticides (Georghiou, 1969; Georghiou, 1986). Many different compounds have been used for insect control including cyclodienes, organophosphates, carbamates, synthetic pyrethroids, insect hormone analogues and insect toxins (Hutson and Roberts, 1985; Oppenoorth, 1985). Resistance to particular insecticides may be conferred by various of mechanisms that each contribute differing degrees of resistance; for example minor resistance can be conferred by reduced penetration of the insecticide, while greater levels can be conferred by target site modifications or increased detoxication.

The next section focuses on OPs, the most widely used class of insecticides. Particular reference is given to malathion, an OP of economic and social importance because of its low mammalian toxicity and extensive use to control disease vectors such as *Anopheles* mosquitoes. Finally, the data on insecticide resistance in *Lucilia cuprina* is summarised and the aims of this study outlined.

1.2 Mode of Action of OP Insecticides

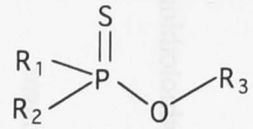
Organophosphorus insecticides (OPs) have been used for insect control since 1944 when parathion was first recognised for its insecticidal activity. A wide range of structures can be placed around the active phosphorus centre of OPs (Figure 1.1; Table 1.1) and these generate the broad range of physical properties, stability and selectivity that have enabled the effective use of OPs for the past five decades to control a variety of insect species. OPs are esters and, as such, are potentially substrates for esterase enzymes. Although they bind to the catalytic site of many esterases, the rate of hydrolysis is generally very low and in many cases they inhibit the esterase activity. It is by binding to acetylcholinesterase that they elicit their insecticidal effect (O'Brien, 1961; Hutson and Roberts, 1985).

Acetylcholinesterase (EC 3.1.1.7) is one of the components of the cholinergic system, which comprises the synaptic transmitter, acetylcholine (ACh), the acetylcholine synthesizing enzyme, choline acetyltransferase or choline acetylase (ChA), and the acetylcholine hydrolysing enzyme, acetylcholinesterase (AChE) (Smallman and Mansingh, 1969; Hutson and Roberts, 1985). ACh is hydrolysed by AChE into the inactive acetic acid and choline, allowing the original state of the postsynaptic cleft to be re-established (Eto, 1974; Hutson and Roberts, 1985). Inhibition of AChE prevents the repolarisation of the nerve cell, causing continuous firing of the nerve and eventually death (Figure 1.2).

Insect AChE appears to be very similar to vertebrate AChE (Hall and Spierer, 1986; Fournier *et al.*, 1988a; Fournier *et al.*, 1989; Hall and Malcolm, 1991). The protein is restricted to the nervous system, has a substrate specificity between those of vertebrate AChE and butylcholinesterase (EC 3.1.1.8) (Gnagey *et al.*, 1987) and, in many species, is composed of a single molecular form which is a glycosylated dimer attached to the membrane via a glycolipid anchor (Fournier *et al.*, 1988b; Hall and Malcolm, 1991). The active-site of AChE contains the catalytic triad typical of α/β hydrolase fold enzymes: nucleophile (serine), histidine and acid (glutamic acid) (Ollis *et al.*, 1992). The catalytic triad is contained within an active site gorge, a substantial proportion of which is lined with aromatic amino acid residues. It is thought that the aromatic residues, highly conserved across species, may act to guide the ACh into the active site within the gorge (Maelicke, 1991). Electron transfer through the catalytic triad is an essential part of the mechanism of ester hydrolysis. A negatively charged residue (glutamic acid for AChE) draws a hydrogen atom from an adjacent histidine residue, which in turn draws a hydrogen atom from the active-site serine. This activates the serine, promoting a nucleophilic attack on the carbonyl carbon of ACh and resulting in an acylated enzyme intermediate (Figure 1.3 and refer to Figure 1.6). The final step in hydrolysis requires a residue close to the active-site to activate a water molecule. The activated water molecule attacks the bound acid moiety, acetic acid, activating it and transforming the resulting complex into a transition state with a high energy level. The covalent bond is subsequently hydrolysed, acetic acid released and energy in the system is minimised (Soreq *et al.*, 1992; Figure 1.3).

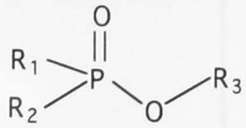
OPs mimic the action of ACh in respect to their interaction with AChE (Hutson and Roberts, 1985; Figure 1.2). In place of the acyl radical being transferred to form the intermediate acylated enzyme, OP compounds transfer a phosphoryl radical and phosphorylate the serine hydroxl group. The phosphorylated enzyme is hydrolysed very slowly and so the enzyme is effectively inhibited (Wilson, 1951; Hutson and Roberts, 1985). Although the bound OP configuration resembles the transition state of ACh

a.



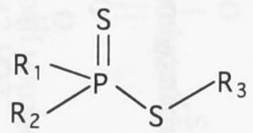
Phosphorothionate

b.



Phosphate

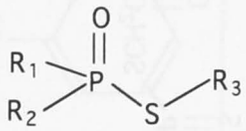
c.



Phosphorothiolothionate

(Phosphorodithioate)

d.



Phosphorothiolate

Figure 1.1 Basic OP structure around the phosphorus group.

a. Phosphorothionate (eg. chlorpyrifos, diazinon, parathion & propetamphos)

b. Phosphate (eg. chlorpyrifos oxon, diazoxon, paraoxon & propetamphos oxon)

c. Phosphorothiolothionate (eg. acethion, dimethoate, formothion, malathion & phenthoate)

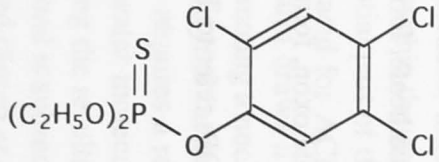
d. Phosphorothiolate (eg. aceoxon, dimethoate oxon, formoxon, malaoxon & phenthoate oxon)

For the complete structures of each of these OPs refer to Table 1.1

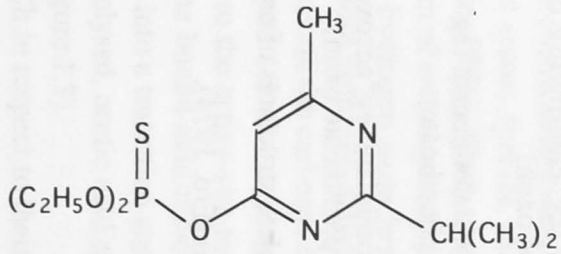
Adapted from (Eto, 1974).

Table 1.1 The Structures of the OPs referred to in the text[a].

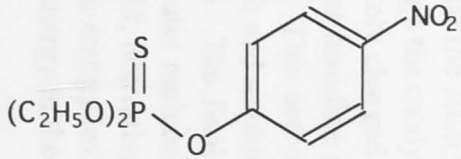
Chlorpyrifos
Diethyl 3,5,6-trichloro-2-pyridyl phosphorothionate



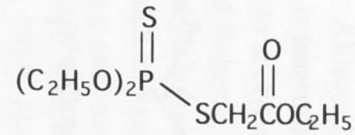
Diazinon
Diethyl 2-isopropyl-6-methyl-4-pyrimidinyl phosphorothionate



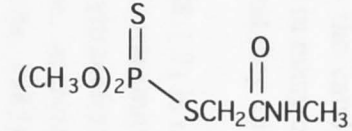
Parathion
Diethyl *p*-nitrophenyl phosphorothionate



Acethion
Diethyl *S*-(ethoxycarbonylmethyl) phosporothiolothionate



Dimethoate
Dimethyl *S*-(N-methylcarbamoylmethyl) phosporothiolothionate



Formothion
Dimethyl *S*-(N-formyl-N-methylcarbamoylmethyl) phosphorothiolothionate

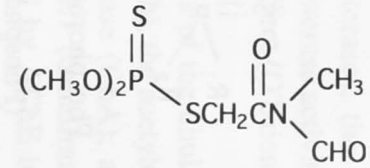
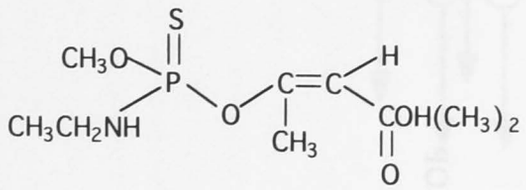


Table 1.1 continued

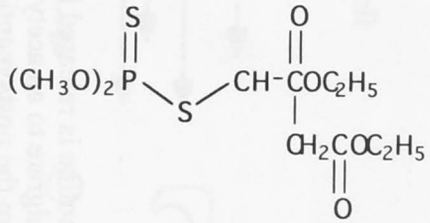
Propetamphos

(*E*)-2-isopropoxycarbonyl-1-methylvinylmethylethyl phosphoramidothioate



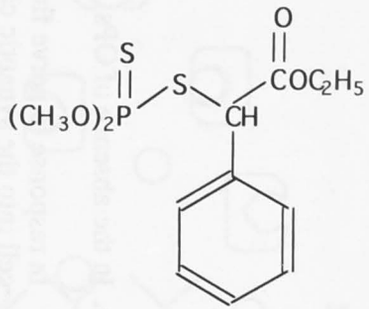
Malathion

S-[1,2-di(ethoxycarbonyl)ethyl] dimethyl phosphorothiolothionate



Phenthoate

Dimethyl *S*-[(α-ethoxycarbonyl)benzyl] phosphorothiolothionate



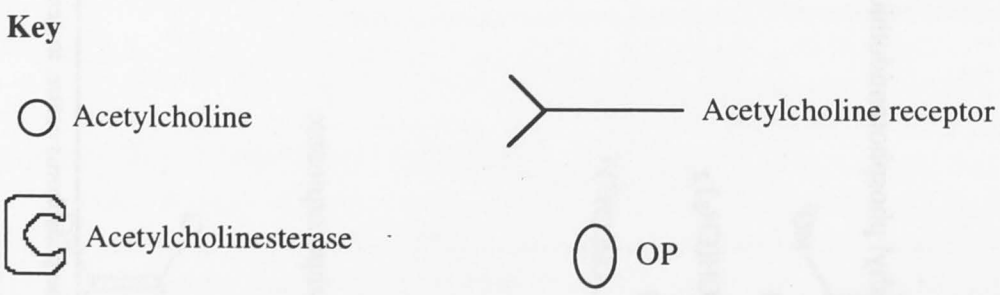
[a]Substitute an oxgen for the sulphur doubled bonded to the phosphorus centre, to generate the oxygen anaologue of each of these OPs.

Figure 1.2 **a.** In the absence of OPs

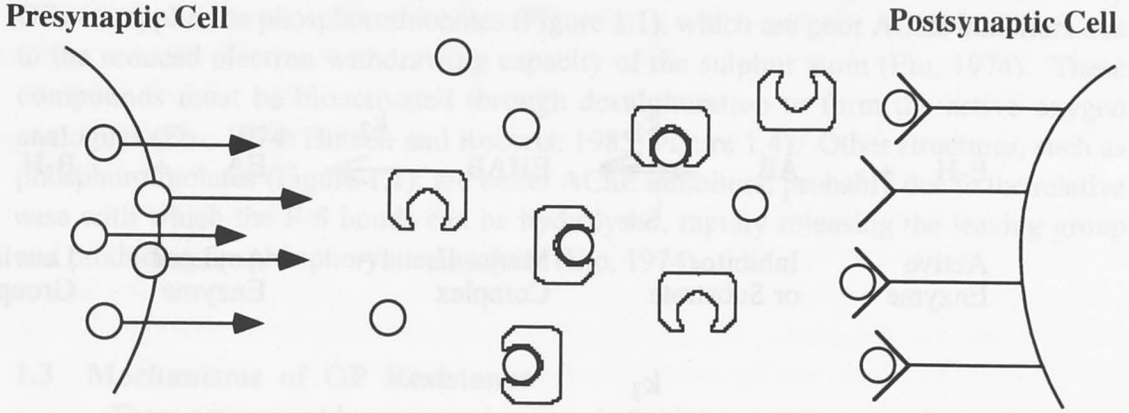
In response to nerve firing, acetylcholine is released from a presynaptic cell into the synaptic cleft. It can migrate to an acetylcholine receptor and intiate nerve firing from the postsynaptic cell. Acetylcholinesterase hydrolsyses acetylcholine, which allows repolarisation of the postsynaptic cell and terminates the nerve signal.

b. In the presence of OPs

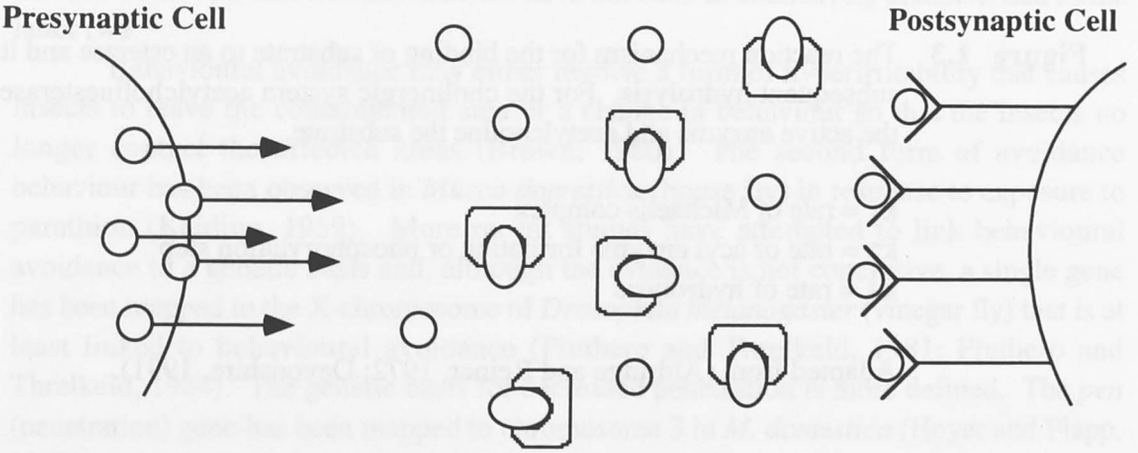
OPs bind to acetylcholinesterase and prevent repolarisation of the postsynaptic cell, causing continuous firing of the nerve and eventual death.



a.


Presynaptic Cell
Postsynaptic Cell

b.


Presynaptic Cell
Postsynaptic Cell

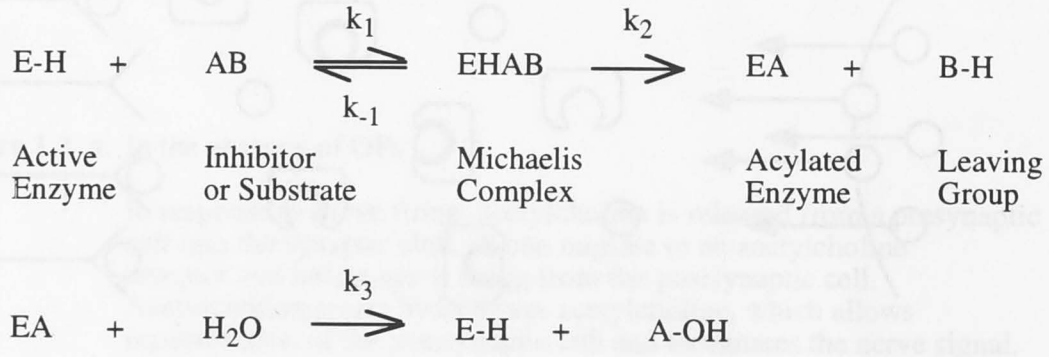


Figure 1.3 The reaction mechanism for the binding of substrate to an esterase and its subsequent hydrolysis. For the cholinergic system acetylcholinesterase is the active enzyme and acetylcholine the substrate.

k_1 = rate of Michaelis complex
k_2 = rate of acyl enzyme formation or phosphorylation step
k_3 = rate of hydrolysis

Adapted from (Aldridge and Reiner, 1972; Devonshire, 1991).

hydrolysis, the OP configuration is stable, not activated. Therefore, hydrolysis of the OP-enzyme complex is much slower than that of acetyl-enzyme (lower k_3 relative to that for ACh). Indeed, for some OPs phosphorylation of the active-site serine is essentially irreversible (Soreq *et al.*, 1992). The electrophilic nature of the OP phosphorus atom is central to its mechanism of action, conferring the ability to phosphorylate the serine hydroxyl group in the AChE active site. The anti-acetylcholinesterase activity of the OP depends on the phosphorylating ability of its phosphoester linkage (Eto, 1974). Many OPs are applied as phosphorothionates (Figure 1.1), which are poor AChE inhibitors due to the reduced electron withdrawing capacity of the sulphur atom (Eto, 1974). These compounds must be bioactivated through desulphuration to form the active oxygen analogues (Eto, 1974; Hutson and Roberts, 1985; Figure 1.4). Other structures, such as phosphorothiolates (Figure 1.1), are better AChE inhibitors, probably due to the relative ease with which the P-S bonds can be hydrolysed, rapidly releasing the leaving group and producing the phosphorylated enzyme (Eto, 1974).

1.3 Mechanisms of OP Resistance

There are several known mechanisms by which insects may evolve resistance to OPs, and these are outlined below. When considering each mechanism separately it is important to note that some insect species have evolved more than one of the following resistance mechanisms to particular OPs, and that these mechanisms may confer cross-resistance to other OPs. Two mechanisms generally confer lower levels of resistance, behavioural avoidance and decreased penetration, and two further resistance mechanisms tend to confer higher levels of resistance, OP insensitive AChE and increased OP detoxication. The first two mechanisms have not been as extensively characterised as the latter two.

Behavioural avoidance may either involve a form of hyperirritability that causes insects to leave the contaminated area or a change in behaviour so that the insects no longer contact the affected areas (Brown, 1960). The second form of avoidance behaviour has been observed in *Musca domestica* (house fly) in response to exposure to parathion (Keiding, 1959). More recent studies have attempted to link behavioural avoidance to a genetic basis and, although the evidence is not conclusive, a single gene has been mapped to the X-chromosome of *Drosophila melanogaster* (vinegar fly) that is at least linked to behavioural avoidance (Pluthero and Threlkeld, 1981; Pluthero and Threlkeld, 1984). The genetic basis for decreased penetration is more defined. The *pen* (penetration) gene has been mapped to chromosome 3 in *M. domestica* (Hoyer and Plapp, 1968), and appears to confer a low level of resistance to a broad range of insecticides in conjunction with metabolic resistance (Oppenoorth, 1985). Investigations into the biochemical basis have suggested that decreased penetration may result from an increase in the cuticular phospholipid concentration (Patil and Guthrie, 1979). Both of these mechanisms may play a minor but important role in the adaptive response of insects to the presence of insecticides and enhance resistance in strains with metabolic resistance mechanisms (Oppenoorth, 1985).

1.3.1 Acetylcholinesterase Insensitivity

Insensitive AChE is an important resistance mechanism to OPs and has been reported in at least six insect orders (Table 1.2). Insect AChE is encoded by a single gene and OP-insensitive AChE is an allelic variation of the OP-susceptible enzyme (Iwata and Hama, 1972; Devonshire, 1975; Plapp and Tripathi, 1978; Brown and Bryson, 1992; Fournier *et al.*, 1992a; Pralavorio and Fournier, 1992). In several species, the altered AChE appears to have a reduced affinity for OPs but there is no change in kinetic parameters for ACh (Raymond *et al.*, 1985; Zhu and Brindley, 1990; Bonning and Hemingway, 1991). However, changes in K_M (a measure of the affinity of the enzyme for the substrate), though not the V_{max} (a measure of the rate at which the enzyme hydrolyses the substrate), have been found in two resistant *M. domestica* strains compared to a susceptible strain (Devonshire and Moores, 1984). One resistant strain (arD) was reported to have a higher K_M (reduced affinity) for ACh and lowered K_Is for OPs, indicating that the OPs did not bind as readily to this enzyme as to the susceptible enzyme. The second resistant strain (Fm22) had an increased affinity for ACh (Devonshire and Moores, 1984), suggesting that any ACh present in synapses would bind more readily to this altered AChE and protect it from inhibition by OPs.

Table 1.2 Examples of species with altered acetylcholinesterase enzymes as an organophosphate insecticide resistance mechanism.

Species	Reference
Coleoptera:	
Leptinotarsa decemlineata	Wierenga and Hollingworth, 1993
Diptera:	
Anopheles albimanus	Hemingway and Georghiou, 1983
Culex pipiens	Raymond *et al.*, 1985; Bonning and Hemingway, 1991
Culex quinquefaciatus	Bisset *et al.*, 1990; Tang *et al.*, 1990
Drosophila melanogaster	Fournier *et al.*, 1992a; Morton and Singh, 1982; Mutero *et al.*, 1994
Musca domestica	Devonshire, 1975; Devonshire and Sawicki, 1975; Oppenoorth *et al.*, 1977; Oppenoorth, 1982; Devonshire and Moores, 1984
Heteroptera:	
Lygus hesperus	Zhu and Brindley, 1990; Zhu and Brindley, 1992
Nepthottettix cincticeps	Iwata and Hama, 1972; Hama, 1983
Homoptera:	
Bemisia tabaci	Byrne and Devonshire, 1991; Byrne and Devonshire, 1993; Byrne *et al.*, 1994
Lepidoptera:	
Heliothis virescens	Brown and Bryson, 1992
Thysanoptera:	
Frankliniella occidentalis	Zhao *et al.*, 1994

In both *D. melanogaster* and *M. domestica* single point mutations in the gene encoding AChE have been found to cause resistance to OPs (Fournier *et al.*, 1992a; Devonshire and Williamson, 1993). In *D. melanogaster* the resistant enzyme shares the same structure as the susceptible enzyme with respect to molecular weight, dimeric structure, hydrophobicity and peptide composition except that it has a replacement of a phenylalanine with a tyrosine at codon position 368 (Fournier *et al.*, 1992a). This phenylalanine corresponds to phenylalanine 290 (Phe_{290}) of *Torpedo californica* (electric ray) AChE. The *Torpedo* AChE has been crystallised (Sussman *et al.*, 1991) and the available three dimensional structure shows that phenylalanine 290 is one of the aromatic amino acids that lines the gorge leading to the catalytic site (Axelsen *et al.*, 1994), and is conserved in all other cholinesterases sequenced, suggesting that it is important for catalytic function.

The structure of human AChE has also been modelled on that of *T. californica* AChE (Ordentlich *et al.*, 1993) revealing that Phe_{297} (equivalent to *Torpedo* Phe_{290} and *D. melanogaster* Phe_{368}) is a size restricting element which determines the substrate specificity of the acyl pocket for the covalent complex (carboxyl group bound to the serine). The mechanism by which this amino acid change causes resistance is unknown but it may prevent bulky substrates such as OPs from entering the gorge while still allowing the entry of smaller molecules like ACh. Four other point mutations have been identified in insensitive AChE isozymes from *D. melanogaster*, the enzymes from the most resistant insects carrying several mutations (Fournier *et al.*, 1993; Mutero *et al.*, 1994). Many of these mutations are also found in OP resistant AChE isozymes from *M. domestica* (A. Devonshire, personal communication).

1.3.2 Increased Insecticide Detoxication

Increased metabolism of OPs generally results in the production of less toxic, more water soluble products via oxidation (mixed function oxidase activity), conjugation (glutathione S-transferase activity) or cleavage (esterase activity) reactions. Each of these reactions can produce identical ester metabolites. In order to distinguish one enzyme system from another at least four criteria need to be satisfied (Motoyama and Dauterman, 1974):

a. the subcellular localisation (mixed function oxidase activity is restricted to the microsomal fractions and glutathione S-transferase activity generally to the cytosol);

b. the co-factor requirements (mixed function oxidase and glutathione S-transferase activities require NADPH and oxygen or glutathione, respectively);

c. the nature of the product (for example, diazoxon, S-(2-isopropyl-4-methyl-6-pyrimidinyl) glutathione or diethyl phosphoric acid); and

d. the response to specific inhibitors (mixed function oxidase activity is inhibited by piperonyl butoxide (PBO; Wilkinson, 1971; Hodgson *et al.*, 1993), and esterases by S,S,S-tributylphosphorotrithioate (TBTP, DEF®) or triphenylphosphate (TPP; Plapp and Tong, 1966).

However, not all metabolism results in less toxic products. Occasionally, derivatives with increased toxicity are produced. For example, the oxygen analogues of most OPs are more toxic than the thionate and these can be produced by desulphuration of the thionate compounds.

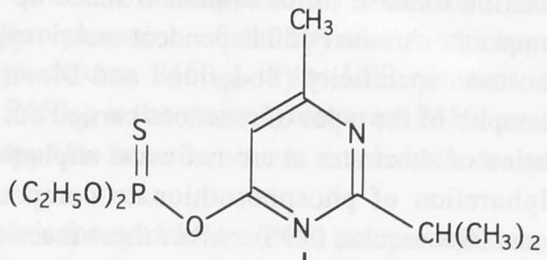
Figure 1.4 Degradation pathway of diazinon by mixed function oxidase activity.

a. Diazinon may be dearylated to produce diethyl phosphorothioic acid and 2-isopropyl-4-hydroxy-6-methyl pyrimidine.

b. Diazoxon is produced by desulphuration. Diazoxon may be further dearylated to produce diethyl phosphoric acid. Refer to Section 1.3.2.1 for details.

Adapted from (Eto, 1974).

Diazinon

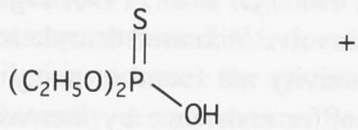


Mixed Function Oxidase

+ NADPH
+ O_2

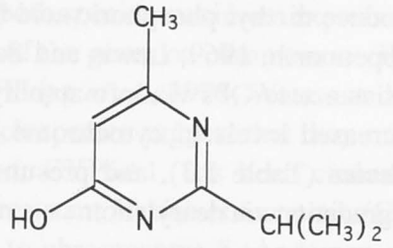
a. Dearylation

(detoxication)



Diethyl Phosphorothioic Acid

+

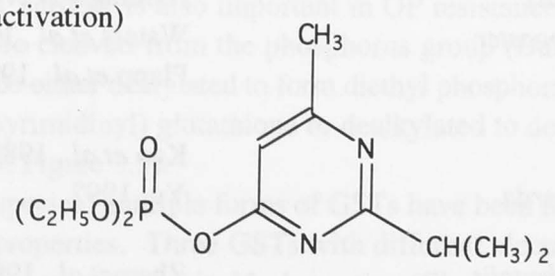


2-isopropyl-4-hydroxy-6-methyl pyrimidine

OR

b. Desulphuration

(activation)



Diazoxon

1.3.2.1 *Mixed Function Oxidase (mfo)*

The mixed function oxidase (mfo) system is made up of several components including the microsomal cytochrome P450 dependent monooxygenases (EC 1.14.14.1), and it has a broad substrate specificity (Soderlund and Bloomquist, 1990; Scott and Wheelock, 1992). Examples of the types of reactions carried out by members of the mfo system are: hydroxylation of substrates at aromatic and aliphatic bonds, epoxidation of double bonds, desulphuration of phosphorothionate compounds and oxidation of sulphides (Soderlund and Bloomquist, 1990). All of these reactions involve an oxidative step and require the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen (Wilkinson, 1983; Hodgson *et al.*, 1993).

The many different types of cytochrome P450s and their broad substrate specificity are believed to underlie their involvement in resistance to a variety of insecticides. Mfo activity is able to catalyse two types of reactions with OPs, dearylation and desulphuration. The first reaction produces less toxic, more water soluble products. For example, diazinon may be dearylated to form diethyl phosphorothioic acid (Nakatsugawa *et al.*, 1969; Dahm, 1970; Eto, 1974; Oi *et al.*, 1990) and diazoxon to produce diethyl phosphoric acid in microsomal fractions of *M. domestica* (ElBashir and Oppenoorth, 1969; Lewis and Sawicki, 1971; Oi *et al.*, 1990; Figure 1.4). Therefore resistance to OPs via mfo activity may involve increased dearylation activity. Indeed, increased levels of cytochrome P450 activity are found in many OP resistant insect species (Table 1.3), and presumably confer resistance by increasing the rate of OP degradation via dearylation.

Table 1.3 Examples of species with organophosphate insecticide resistance due to increased cytochrome P450 activity.

Species	Reference
Coleoptera:	
Leptinotarsa decemlineata	Ahammad-Sahib *et al.*, 1994
Tribolium castaneum	Dyte, 1972; Cohen, 1982
Diptera:	
Anopheles subpictus	Hemingway *et al.*, 1991
Drosophila melanogaster	Waters *et al.*, 1992a; Waters *et al.*, 1992b
Musca domestica	Plapp *et al.*, 1976
Lepidoptera:	
Plutella xylostella	Kao *et al.*, 1989
Spodoptera frugiperda	Yu, 1992
Thysanoptera:	
Frankliniella occidentalis	Zhao *et al.*, 1994

The second mfo-mediated reaction with OPs involves activation of the thionate OP compound through desulphuration to produce the more toxic oxygen analogue. For example, diazinon may be desulphurated to produce diazoxon (Figure 1.4). Thus resistance may also be conferred by reduced activation of the thionate to the oxygen analogue. *Blatella germanica* (German cockroach; Siegfried *et al.*, 1990), *Heliothis virescens* (tobacco budworm; Konno *et al.*, 1989), *M. domestica* (Hatano and Scott,

1993), and *Schizaphis graminum* (greenbug; Siegfried and Ono, 1993) all show decreased levels of oxidative desulphuration in resistant strains. In *M. domestica*, reduced oxidative desulphuration of chlorpyrifos to chlorpyrifos oxon is caused by reduced levels of cytochrome $P450_{lpr}$ in the LPR resistant strain (Hatano and Scott, 1993). Cytochrome $P450_{lpr}$ is the major cytochrome P450 involved in desulphuration of chlorpyrifos to chlorpyrifos oxon but is not involved in dearylation of chlorpyrifos or chlorpyrifos oxon.

The genetic basis for cytochrome P450 induced resistance is polygenic in both *D. melanogaster* and *M. domestica*, the two species studied in detail. Two genes on chromosome 3R and another on chromosome 2R in *D. melanogaster* are apparently involved in increased cytochrome P450 expression and segregate with resistance (Hällström, 1985; Hällström and Blanck, 1985; Houpt *et al.*, 1988; Waters and Nix, 1988). Genes involved in cytochrome P450 expression and resistance in *M. domestica* are on the homologous chromosomes to chromosomes 2R and 3R in *D. melanogaster*, chromosomes 2 and 5, respectively (Weller and Foster, 1993). There are two such genes on chromosome 2 and another on chromosome 5 (Oppenoorth, 1967; Plapp and Casida, 1969; Plapp *et al.*, 1976; Hodgson *et al.*, 1993). In both species maximum expression of cytochrome P450 activity is *trans* regulated by one of the genes on chromosome 3R (*D. melanogaster*) or chromosome 2 (*M. domestica*) (Plapp *et al.*, 1976; Waters and Nix, 1988). The similarities between the systems in each species suggest that they may be homologous. Recently, a cytochrome P450 gene, CYP6A1, has been cloned and characterised and it is constitutively expressed in a resistant *M. domestica* strain (Cariño *et al.*, 1992; Cariño *et al.*, 1994). The gene maps to chromosome 5 (Andersen *et al.*, 1994), but the constitutive expression is controlled by a gene on chromosome 2 (Cariño *et al.*, 1994).

1.3.2.2 *Glutathione S-Transferases (GST)*

Glutathione S-transferases (GSTs; EC 2.5.1.18) catalyse the nucleophilic attack of the tripeptide glutathione (GSH) on a variety of substrates. The sulphur atom of GSH provides electrons for the nucleophilic attack. The endogenous substrates may include epoxides, organic hydroperoxides or activated alkenals resulting from oxidative metabolism (Mannervik, 1985). GSTs can ultimately protect tissues from oxidative damage or stress. GST activity is also important in OP resistance where either aryl or alkyl substituents can be cleaved from the phosphorus group (Dauterman, 1985). For example, diazinon can be either dearylated to form diethyl phosphorothioic acid and S-(2-isopropyl-4-methyl-6-pyrimidinyl) glutathione or dealkylated to desethyl diazinon (Eto, 1974; Dauterman, 1983; Figure 1.5).

In many insect species, multiple forms of GSTs have been reported, with distinct physical and catalytic properties. Three GSTs with different electrophoretic mobilities and substrate specificities were found in *M. domestica* (Clark *et al.*, 1984). Fournier *et al.* (1992b) found subsequently that *M. domestica* has at least two distinct classes of GSTs, GST1 and GST2, that have different molecular weights, are serologically distinct and do not form dimers. Additionally, polyclonal antibodies raised to each class do not cross react (Fournier *et al.*, 1992b). At least two different GST isozymes have also been reported from the mosquitoes, *Aedes aegypti*, *Anopheles freeborni* (Grant *et al.*, 1991; Grant and Hammock, 1992) and *Anopheles gambiae* (Prapanthadara *et al.*, 1993), and also from ~~*Heliothis zea*~~ Helicoverpa zea (corn earworm), *Heliothis virescens*, *Spodoptera frugiperda* (fall armyworm), *Trichoplusia ni* (cabbage looper; Yu, 1989), *Plutella xylostella*

Figure 1.5 Degradation pathway of diazinon by glutathione S-transferase activity.

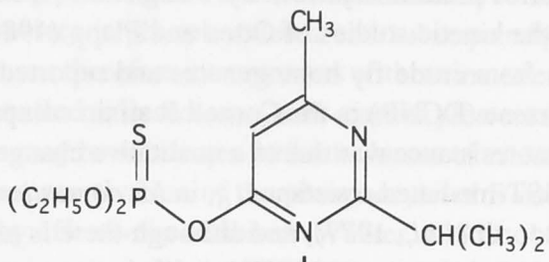
a. Diethyl phosphorothioic acid and S-(2-isopropyl-4-methyl-6-pyrimidinyl)glutathione are produced by dearylation.

b. Desethyl diazinon and S-ethyl glutathione are produced by dealkylation.

Diazoxon may be substituted for diazinon to produce
a. diethyl phosphoric acid or
b. desethyl diazoxon.

Adapted form (Eto, 1974).

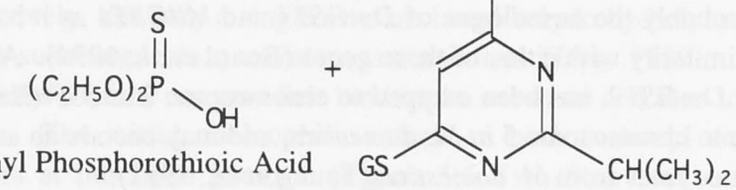
Diazinon



Glutathione S-Transferase

+ Glutathione

a. Dearylation

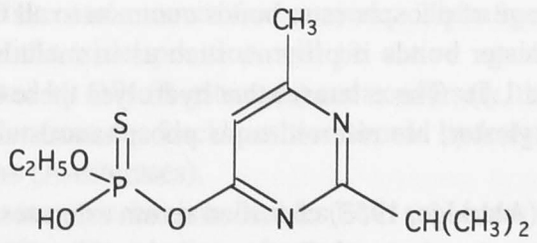


Diethyl Phosphorothioic Acid

S-(2-isopropyl-4-methyl-6-pyrimidinyl)glutathione

OR

b. Dealkylation



S-Ethyl Glutathione

Desethyl Diazinon

(diamondback moth; Chiang and Sun, 1993), and *Tribolium castaneum* (red flour beetle; Reidy *et al.*, 1990).

Cloning of the gene encoding a GST1 isozyme from *M. domestica* showed elevated levels of GST1 mRNA and protein in the Cornell R resistant strain (Fournier *et al.*, 1992b). This supported preliminary work by Wang *et al.* (1991) with the same strain but was in contrast to the kinetic studies of Ottea and Plapp (1984). The latter authors analysed GST kinetics from crude fly homogenates and reported lower K_M values for 1,2-dichloro-4-nitrobenzene (DCNB) in the Cornell R strain compared to the susceptible strain and concluded that resistance was due to a qualitative change in GST activity. The gene associated with GST-mediated resistance, *g*, in *M. domestica* has been localised to chromosome 2 (Oppenoorth *et al.*, 1977), and although there is no direct evidence, the cloned GST1 gene from *M. domestica* (*MdGST1*) is likely to map to the *g* gene.

MdGST1 is 84% identical to the *Drosophila* gene *DmGST1* at the amino acid level (Toung *et al.*, 1990; Wang *et al.*, 1991; Fournier *et al.*, 1992b). The *DmGST1* gene maps to chromosome 3R (at region 87B 8-9; Parkes *et al.*, 1993) the homologous chromosome to *M. domestica* chromosome 2 (Foster *et al.*, 1981; Weller and Foster, 1993) and is probably homologous to *MdGST1* based on sequence identity, activity levels and map position. A GST gene has recently been cloned from *Lucilia cuprina* and it is also probably the homologue of *DmGST1* and *MdGST1* as it has greater than 81% sequence similarity with either of these genes (Board *et al.*, 1994). A second *Drosophila* GST gene, *DmGST2*, has been mapped to chromosome 2 at 53F (Beall *et al.*, 1992) the homologue to chromosome 5 in *M. domestica*, and may encode an analogous enzyme to the GST2 isozymes from *M. domestica* (Toung *et al.*, 1991).

1.3.2.3 *Esterases*

Insect esterases exist in multiple forms and can be distinguished after separation by electrophoresis using artificial histochemical substrates such as esters of *p*-nitrophenol and α- and β-naphthol. Fifteen distinct esterases have been resolved in adult *M. domestica* (Narang *et al.*, 1976; Maa and Terriere, 1983) and 23 in *D. melanogaster* (Healy *et al.*, 1991; Spackman *et al.*, 1994). OP insecticides may be cleaved by esterases in two ways: by the cleavage of phosphoester bonds common to all OPs (Table 1.1) or by the cleavage of carboxylester bonds if present, such as in malathion, phenthoate and related compounds (Table 1.1). The esterases that hydrolyse these two classes of bonds, phosphoester and carboxylester, are referred to as phosphatases and carboxylesterases respectively.

In 1953 Aldridge (Aldridge, 1953) classified serum esterases of many species into two distinct groups: A-esterases and B-esterases. A-esterases were originally distinguished by their insensitivity to paraoxon and ability to hydrolyse *p*-nitrophenyl acetate at a higher rate than *p*-nitrophenyl butyrate. B-esterases were classified as being inhibited by paraoxon and hydrolysing *p*-nitrophenyl butyrate at the same rate or faster than *p*-nitrophenyl acetate. With the purification and characterisation of several esterases in each class, the classifications have been refined but esterases essentially still fall into these two broad groups. A-esterases are now classified as a large group that includes esterases that hydrolyse carboxylic esters, carbamic esters and esters of phosphoric acids and are not inhibited in progressive reaction by OPs and other acylating agents (Aldridge, 1993). They cleave phosphoester bonds and alternative names include arylesterases, phosphatases or phosphorotriester hydrolases. For example, rabbit and human serum paraoxonases are able to hydrolyse the active oxygen analogues of chlorpyrifos and

parathion (Furlong *et al.*, 1989; Furlong *et al.*, 1991; Gan *et al.*, 1991) and are classified as A-esterases.

An esterase is classified as a B-esterase if it is inhibited in a progressive and temperature dependent reaction by OPs. This has been shown using esterases with a serine hydroxyl group in the active site (serine esterases) to involve an intermediate step where the serine moiety is phosphorylated and subsequently hydrolysed (Harel *et al.*, 1991). B-esterases are therefore synonymous with serine esterases (Aldridge, 1993) and have also been called aliesterases and carboxylesterases (Oppenoorth, 1985). Paradoxically, OPs may be substrates for both classes of esterase. OPs phosphorylate B-esterases. However, reactivation of the phosphorylated B-esterases occurs very slowly and they are thereby effectively inhibited (Walker, 1993). AChE is an example of a B-esterase and, as mentioned above (Section 1.2), it is inhibited by OPs via phosphorylation of the serine residue in the active site. It is important to note that in aqueous solutions, most phosphorylated enzymes may be reactivated, albeit slowly, by various nucleophiles (e.g. water molecules) that detach the OP from the serine hydroxyl group (Harel *et al.*, 1991).

Two types of mechanisms involving either a two step or single step reaction have been proposed to explain why OPs can either act as substrates for or inhibit A- and B-esterases, respectively, and why a single esterase is unlikely to hydrolyse both carboxyl and phosphoryl substrates via a covalent intermediate (Järv, 1989). The two step mechanism requires that the enzyme first bind to the substrate and then to the activated water molecule. The initial nucleophilic attack on both carboxyl and phosphoryl substrates occurs at the same position (Figure 1.6 Two Step Mechanism, Step 1), displacing the leaving group and forming a covalent intermediate with the enzyme at the active site (Figure 1.6 Two Step Mechanism, Step 2). The positioning of the substrate within the active site centre influences the direction of the second nucleophilic attack by the activated water molecule. Because of the arrangement of the *sp* hybrid orbitals of the phosphoryl or carboxyl moiety when bound to the serine and the positioning of the substrate, the activated water can only bind at either the 'side' (carboxyl) or 'back' (phosphoryl) positions (Figure 1.6 Two Step Mechanism, Step 2), to hydrolyse the covalent intermediate and recover free enzyme. Therefore, esterases that catalytically activate water in the 'side' position will be active against carboxylesters and near irreversibly inhibited by OPs (B-esterases) and conversely, those that catalytically activate the water molecular from the 'back' position should hydrolyse OPs, but near irreversibly bind carboxylesters (A-esterases).

The single step mechanism enables both substrates to be hydrolysed by the one enzyme (Järv, 1989). The binding of an activated water molecule to the centre of the esterase before binding to the substrate, would enable the release of both products after substrate binding in a single step (Figure 1.6 Single Step Mechanism). The activation of a water molecule in the esterase centre could be achieved via some basic functional groups or metal ions. Mammalian paraoxonases require divalent cations for activation (Furlong *et al.*, 1989; Furlong *et al.*, 1991; Gan *et al.*, 1991) and may hydrolyse OPs by this single step mechanism (Aldridge, 1993).

Figure 1.6 The different mechanisms of hydrolysing carboxyl and phosphoryl groups.

A Two Step Mechanism (eg. Carboxylesterases)

Step 1. Nucleophilic substitution reactions at the
a. carboxyl or
b. phosphoryl groups.
X = leaving group Nü = attacking nucleophile
The arrow indicates the direction of the initial nucleophilic attack.

Step 2 The mechanism of hydrolysis of
a. acyl- or
b. phosphoryl- enzymes.
The activated water molecules can only bind to the 'side' of the acylated enzyme intermediate or 'back' position of the phosphoryl enzyme intermediate due to the orientation of the *sp* hybrid orbitals and postioning of the substrate.
The arrow indicates the direction of the second nucleophilic attack by the activated water molecule.

Single Step Mechanism (eg. A-esterases)

The direct displacement mechanism for
a. carbonyl or
b. phosphoryl groups involving the activated water molecule.

Adapted from (Järv, 1989).

Two Step Mechanism

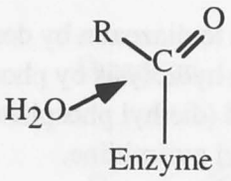
Step 1.

a.

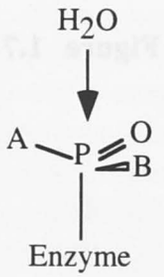
b.

Step 2.

a.

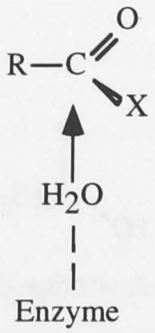


b.



Single Step Mechanism

a.



b.

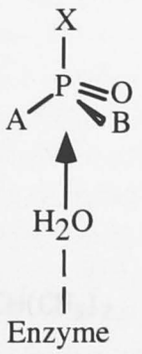
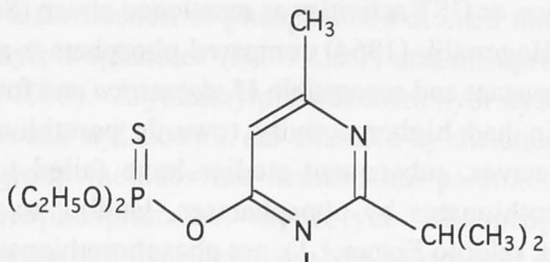


Figure 1.7 Degradation pathway of diazinon by phosphatase activity.

Diazinon must first be activated to diazoxon by desulphuration via mixed function oxidase activity before hydrolysis by phosphatase activity to produce diethyl phosphoric acid (diethyl phosphate) and 2-isopropyl-4-hydroxy-6-methyl pyrimidine.

Adapted form (Eto, 1974).

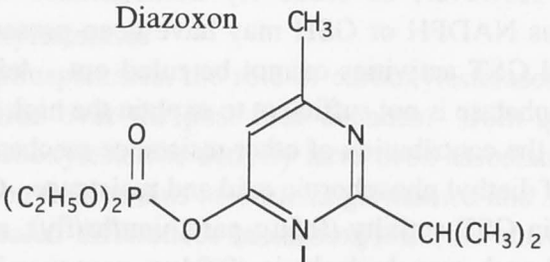
Diazinon



Mixed Function Oxidase

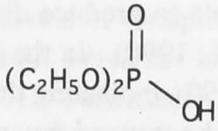
(desulphuration)

Diazoxon



Phosphatase

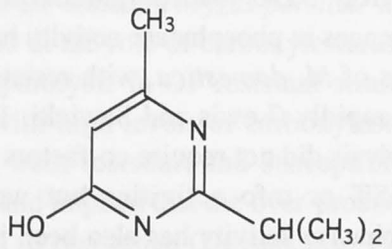
(Hydolysis)



Diethyl Phosphoric Acid

(Diethyl Phosphate)

+



2-isopropyl-4-hydroxy-6-methyl pyrimidine

1.3.2.3.1 Phosphatases

OP hydrolysis by phosphatases can produce either a dialkylphosphoric acid or a desalkyl derivative and an alcohol. Although the role of phosphatases in OP resistance has always been thought to be important, very few studies have actually isolated esterases capable of hydrolysing OPs. Part of the problem in identifying phosphatase activity is that the products from ester cleavage cannot be distinguished from those produced from cytochrome P450 oxidation or GST activities as mentioned above (Section 1.3.2).

Matsumura and Hogendijk (1964) compared phosphatase activities of partially purified enzymes from resistant and susceptible *M. domestica* and found that the enzymes from the resistant strain had higher activity towards parathion than those of the susceptible strain. However, subsequent studies have failed to find evidence for hydrolysis of phosphorothionates by phosphatases, leading to the hypothesis that phosphates (eg. diazoxon, refer to Figure 1.1), not phosphorothionates (eg. diazinon) are the substrates for phosphatases (Welling *et al.*, 1971; Figure 1.7).

Welling *et al.* (1971) reported the presence of a phosphatase able to hydrolyse paraoxon in whole fly homogenates from the E_1 resistant strain of *M. domestica*. The phosphatase enzyme from the E_1 strain is capable of degrading 2.5pmol of paraoxon per minute per fly (Welling *et al.*, 1971) and has a turnover number of 0.2 min^{-1} (Oppenoorth, 1985). However, as crude fly homogenates were used in these experiments, endogenous NADPH or GSH may have been present and therefore the contribution of mfo and GST activities cannot be ruled out. Additionally, the slow turnover rate of the phosphatase is not sufficient to explain the high level of resistance to OPs in the E_1 strain and the contribution of other resistance mechanisms is important to the overall production of diethyl phosphoroic acid and resistance. Comparative rates of paraoxon degradation via GST activity (0.5µg parathion/hr/fly), mfo activity (0.17µg paraoxon/hr/fly) and phosphatase hydrolysis (0.04µg paraoxon/hr/fly; Sawicki and Keiding, 1981) illustrate this point. However, the phosphatase may be the more important resistance mechanism despite the lower turnover rate because it can scavenge OPs at very low concentrations due to its very low K_M ($4x10^{-9}$M for paraoxon; Welling *et al.*, 1971) compared with GST (K_M $>10^{-3}$M for paraoxon; Oppenoorth, 1985) and mfo (K_M $5x10^{-6}$M for paraoxon; ElBashir and Oppenoorth, 1969). Interstrain differences in phosphatase activity have been observed between susceptible and resistant strains of *M. domestica*, with resistant strains able to produce diethyl phosphoric acid more rapidly (Lewis and Sawicki, 1971; Oi *et al.*, 1990). In the latter study, diazoxon hydrolysis did not require co-factors (Oi *et al.*, 1990), indicating that it was not mediated by GST or mfo activities but was likely to be caused by phosphatase activity. Phosphatase activity has also been implicated in the hydrolysis of chlorpyrifos oxon in both *Blatella germanica* (Siegfried *et al.*, 1990) and *Schizaphis graminum* (Siegfried and Ono, 1993).

Phosphatases have been found that require divalent cations for increased activity and OP hydrolysis. A phosphatase purified from *Heliothis virescens* is able to hydrolyse methyl paraoxon and resembles the human, rabbit and rat paraoxonase in its requirement for metal ions (Konno *et al.*, 1989; Konno *et al.*, 1990). The three mammalian enzymes require calcium for activity and are inhibited by EDTA (ethylene dinitrilotetra-acetic acid; Furlong *et al.*, 1989; Furlong *et al.*, 1991; Hassett *et al.*, 1991). These enzymes do not resemble serine esterases and are classified as aryldialkylphosphatases. The *H. virescens* enzyme, on the other hand, requires cobalt or manganese, is not affected by EDTA, and is also probably not a serine esterase because TBTP, known to bind the serine active site,

only partially inhibits both enzyme activity and resistance (Konno *et al.*, 1990). A *M. domestica* esterase has a similar requirement for cobalt or manganese for diisopropylfluorophosphate (DFP) hydrolysis (Krueger and Casida, 1961). However, this enzyme is unable to hydrolyse parathion or paraoxon (Krueger and Casida, 1961), suggesting that it is different from the phosphatase activity observed in the E_1 strain, the first being an A-esterase and the latter a B-esterase.

A recent classification of phosphatases divided this class of enzymes into two groups: aryldialkylphosphatases (EC 3.1.8.1) and diisopropylfluorophosphatases (EC 3.1.8.2) (Reiner, 1993). Aryldialkylphosphatases hydrolyse aryldialkylphosphates into dialkylphosphates and aryl alcohol, are inhibited by chelating agents and require divalent cations. This group includes the mammalian paraoxonases. The second group, diisopropylfluorophosphatases, hydrolyse diisopropylfluorophosphates into diisopropylphosphate and fluoride, hydrolyse phosphorus anhydride bonds and are also inhibited by chelating agents and require divalent cations. These classifications are based on mammalian studies. Although the insect phosphatases mentioned above could be classified as aryldialkylphosphatases because they hydrolyse OPs, they do not share other characteristic features of aryldialkylphosphatases and perhaps belong to a third class of phosphatases.

1.3.2.3.2 Carboxylesterases

As with phosphatases, the role of carboxylesterases in OP resistance has been extensively studied over the past four decades. Both quantitative and qualitative differences in carboxylesterase activity have been associated with OP resistance. For example, assays on field strains of *Blatella germanica* and *Aphis gossypii* (cotton aphid; Homoptera) revealed differences in carboxylesterase activities between chlorpyrifos resistant and susceptible strains that were best explained by the expression of different carboxylesterase isozymes in the resistant compared with the susceptible strains (Hemingway *et al.*, 1993; O'Brien *et al.*, 1992). More detailed studies on OP resistance conferred by qualitative changes in carboxylesterase activities have been reported for malathion carboxylesterase (refer to Section 1.4.5).

The amplification of carboxylesterases in OP resistant *Myzus persicae* and *Culex* mosquitos provide the two best studied examples of the role of carboxylesterases in OP resistance. High levels of α-naphthylacetate hydrolysis in OP resistant strains of the green peach aphid *M. persicae* is associated with high levels of carboxylesterase E4 (Devonshire, 1977). Purification of E4 from both resistant and susceptible strains revealed that they had the same kinetic constants and implied that the over-production of a structurally unaltered E4 was causally involved in resistance (Devonshire, 1977). E4 hydrolysed both α-naphthylacetate and paraoxon, but the turnover rate towards paraoxon and other OPs was very low (k_3 = 0.1 to 3 molecules/hour; refer to Figure 1.3; Devonshire, 1989). However, the amount of E4 present in resistant aphids is so great that a substantial proportion of applied insecticide is detoxified by simply binding to the catalytic site (Devonshire, 1989). Thus the over-production of E4 acts primarily to sequester the OPs and prevent their interaction with AChE.

The amount of E4 enzyme in seven aphid clonal lines formed a geometric series with a factor close to two, with increasing levels of resistance implicating tandem duplication of the E4 locus as the mechanism for the over-production of the E4 enzyme (Devonshire and Sawicki, 1979). The subsequent cloning of the gene permitted the direct demonstration of duplication of the E4 locus in resistant aphid clones (Field *et al.*, 1988).

The cloning of the genes encoding E4 and FE4, a mutant form of E4 with similar hydrolysing activities (it is able to hydrolyse α-naphthylacetate 1.5-fold faster than E4) and a slightly different electrophoretic mobility, showed that both are serine esterases and contain a catalytic triad the same as that found in AChE (Field *et al.*, 1993; Section 1.2).

Carboxylesterases are also amplified in resistant strains of several species of *Culex* mosquitoes. Increased esterase activities are found in *C. pipiens*, *C. quinquefasciatus* and *C. tarsalis*, but different enzymes are overproduced in each species (Mouchès *et al.*, 1987). Two amplified esterases are always found together in *C. quinquefasciatus* on at least three continents (Raymond *et al.*, 1991). In each case studied, the increased esterase activity cannot be separated from OP resistance (Pasteur *et al.*, 1981; Mouchès *et al.*, 1986; Mouchès *et al.*, 1987; Raymond *et al.*, 1987; Wirth *et al.*, 1990; Jayawardena *et al.*, 1994).

The mechanism of OP resistance is the over-production via gene amplification of these carboxylesterases in the resistant strains. In the TEM-R *C. quinquefaciatus* strain, a 70-fold increase in esterase A1 activity corresponded to a 100-fold increase in OP resistance and a 500-fold increase in esterase B1 with an 800-fold increase in resistance (Mouchès *et al.*, 1987). There are at least 250 copies of the gene encoding B1 activity revealing that the increase in activity is the result of gene amplification (Mouchès *et al.*, 1986). The A1 and B1 esterases are encoded by two distinct but closely linked genes on chromosome 3, *Est-3* and *Est-2*, respectively (Pasteur *et al.*, 1981). The inferred amino acid sequence of the B1 esterase reveals regions of similarity to other serine esterases, such as AChE (Mouchès *et al.*, 1990). The A2, B1 and B2 esterases all appear to have little or no interaction with phosphorothionate OPs, are inhibited by the oxygen analogues of these OPs and confer OP resistance via sequestration (Cuany *et al.*, 1993; Karunaratne *et al.*, 1993; Ketterman *et al.*, 1993; Jayawardena *et al.*, 1994). Thus the *M. persicae* and the *Culex* carboxylesterases appear to share many characteristics.

1.3.2.3.3 The Mutant Aliesterase Hypothesis

The mutant aliesterase theory was proposed by van Asperen and Oppenoorth (1959) to explain the relationship between phosphatase activity, lowered carboxylesterase (aliesterase) activity and OP resistance. In several *M. domestica* strains they observed an inverse relationship between total carboxylesterase activity towards artificial substrates and resistance to OPs. Strains that were resistant to OPs expressed lower levels of carboxylesterase activity as assayed using methylbutyrate and α-naphthylacetate than susceptible strains (Oppenoorth and van Asperen, 1960; Oppenoorth and Van Asperen, 1961). The hypothesis proposes that a mutation in gene *a* (for aliesterase activity) results in the change of the enzyme's substrate specificity from carboxylesterase to phosphatase. This transformation of activity corresponds with a decrease in the rate of hydrolysis towards the carboxylesters methylbutyrate or α-naphthylacetate, and an increase in ability to degrade OPs. The hypothesis assumes that the k_3 of the reaction of the enzyme with OPs (Figure 1.3) is increased from virtually nil to 0.2 min^{-1}, using the E_1 phosphatase as an example. In light of the stereochemical considerations of Järv (1989; Section 1.3.2.3) it appears likely that a mutation in an esterase that changes its catalytic function to confer resistance to OPs via hydrolysis of the phosphoester bond will concurrently lose its ability to hydrolyse artificial substrates with carboxylester bonds. Hence, these phosphatases have little or no activity towards α-naphthylacetate.

Not all OP resistant strains of *M. domestica* show esterase activities that support the mutant aliesterase hypothesis (Motoyama and Dauterman, 1974). The *bwb* marker

strain has low aliesterase activity and is susceptible to OPs (Franco and Oppenoorth, 1962), and, on the other hand, there are strains resistant to OPs via other resistance mechanisms (such as an altered AChE, or increased mfo or GST activities) that have normal levels of aliesterase activity (Lewis and Sawicki, 1971; Welling *et al.*, 1971; Oppenoorth, 1985). However, backcross experiments with a parathion resistant strain of *M. domestica* showed that both resistance and low aliesterase activity were inherited together (Oppenoorth and van Asperen, 1960). This result was supported by Franco and Oppenoorth, (1962) when they mapped diazinon resistance and low aliesterase activity (and hence gene *a*) to chromosome 2. To date there is no direct evidence that the mutant enzyme conferring low aliesterase activity in *M. domestica* is a phosphatase, or that it is capable of hydrolysing OPs. The genes conferring OP resistance need to be cloned, sequenced and expressed, or the proteins purified and characterised in order to determine the validity of the mutant aliesterase theory in OP resistance.

1.4 Mechanisms of Malathion Resistance

OP insecticides such as malathion, phenthoate, acethion, dimethoate and formothion have alternative sites for hydrolysis other than the active phosphorus centre, because they also contain carboxylester or carboxylamide linkages (Table 1.1). Specifically, malathion contains two carboxylester bonds in addition to a phosphorodithioate linkage and thus there are at least five sites at which malathion can be hydrolysed (Figure 1.8). Like other OPs, malathion may be activated via mfo desulphuration to produce highly toxic malaoxon. Malaoxon may then undergo similar degradation processes as those outlined for diazoxon, involving mfo dearylation, GST dearylation or dealkylation or phosphatase hydrolysis (Figures 1.4, 1.5 and 1.7). Malathion may also be degraded without undergoing desulphuration by hydrolysis at the carboxylester linkages to produce the non-toxic α and β malathion monocarboxylic acids. The multiple sites for malathion hydrolysis result in numerous potential mechanisms of resistance, each of which will be discussed below. The first four, AChE insensitivity, mfos, GST and phosphatase activities, will be mentioned only briefly with reference to malathion, as they have been outlined for other OPs (Section 1.3).

1.4.1 Insensitive AChE

Phosphorothiolates, such as malaoxon, are generally more active as AChE inhibitors than the corresponding phosphates, presumably because the P-S bond is more easily hydrolysed than the P-O bond (Eto, 1974), and may increase the rate at which the enzyme is phosphorylated (Section 1.2). Many insect species are resistant to malathion via an altered AChE that is less sensitive to inhibition by malaoxon (Table 1.4). Although insensitive AChE appears to be an important resistance mechanism, it is often found in conjunction with hydrolytic mechanisms of resistance. Of the species noted in Table 1.4, all but *Anopheles sacharovi* have hydrolytic mechanisms of resistance in addition to insensitive AChE.

Figure 1.8 Degradation pathway for malathion.

Malathion may either be desulphurated to produce malaoxon or hydrolysed at the carboxylester bonds directly by MCE activity to produce α and β malathion monocarboxylic acids.

Malaoxon may be further degraded by mixed function oxidase or phosphatase activities or by MCE activity at the carboxylester bonds.

Adapted form (Eto, 1974).

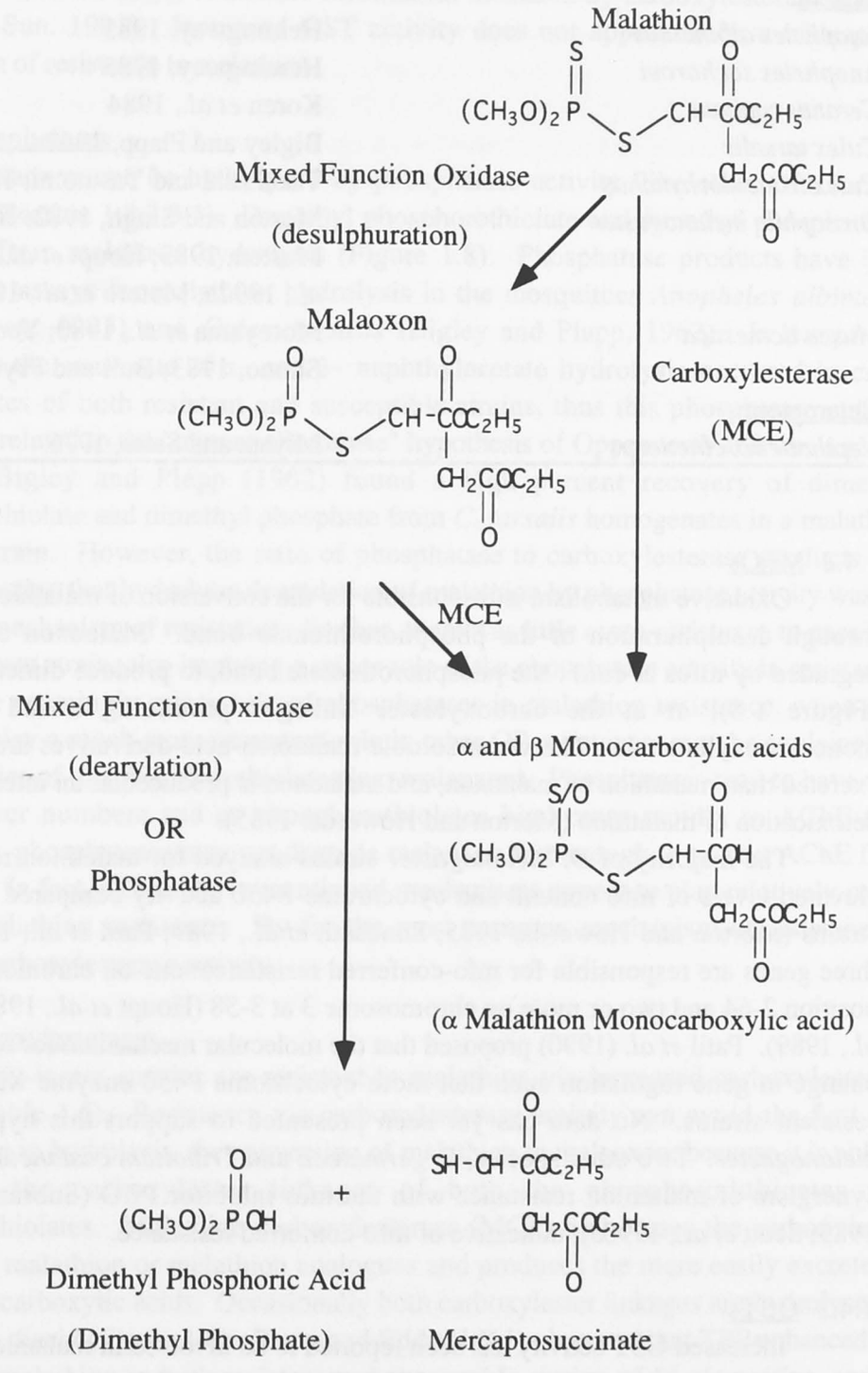

Malathion
Mixed Function Oxidase
(desulphuration)
Malaoxon
Carboxylesterase
(MCE)
MCE
Mixed Function Oxidase
(dearylation)
OR
Phosphatase
α and β Monocarboxylic acids
(α Malathion Monocarboxylic acid)
Dimethyl Phosphoric Acid
(Dimethyl Phosphate)
Mercaptosuccinate

Table 1.4 Examples of species with altered acetylcholinesterase enzymes as a malathion resistance mechanism.

Species	Reference
Diptera:	
Anopheles albimanus	Hemingway, 1985
Anopheles sacharovi	Hemingway, 1985
Ceratitis capitata	Koren *et al.*, 1984
Culex tarsalis	Bigley and Plapp, 1962
Culex tritaeniorhynchus	Takahashi and Yasutomi, 1987
Drosophila melanogaster	Morton and Singh, 1982; Holwerda and Morton, 1983; Houpt *et al.*, 1988; Fournier *et al.*, 1992a; Mutero *et al.*, 1994
Musca domestica	Motoyama *et al.*, 1980; Yeoh *et al.*, 1981; Shono, 1983; Bull and Pryor, 1990
Heteroptera:	
Nepthottettix cincticeps	Miyata and Saito, 1976

1.4.2 MFOs

Oxidative metabolism is responsible for the conversion of malathion to malaoxon through desulphuration of the phosphorothionate bond. Malaoxon can be further degraded by mfos at either the phosphorothiolate bond, to produce dimethyl phosphate (Figure 1.8), or at the carboxylester linkages producing α and β malaoxon monocarboxylic acids. The water soluble malaoxon-acid derivatives are more rapidly excreted than malathion or malaoxon, and malaoxon is produced as an intermediate in the detoxication of malathion (Morton and Howerda, 1985).

The majority of *D. melanogaster* strains assayed for malathion resistance have elevated levels of mfo content and cytochrome P450 activity compared to susceptible strains (Morton and Howerda, 1985; Sundseth *et al.*, 1989; Patil *et al.*, 1990). At least three genes are responsible for mfo-conferred resistance: one on chromosome 2 at map position 2-64 and two or more on chromosome 3 at 3-58 (Houpt *et al.*, 1988; Sundseth *et al.*, 1989). Patil *et al.* (1990) proposed that the molecular mechanism for resistance was a change in gene regulation such that more cytochrome P450 enzyme was produced in resistant strains. No data has yet been presented to support this hypothesis in *D. melanogaster*. Two other species, *B. germanica* and *Tribolium castaneum*, have shown synergism of malathion resistance with the mfo inhibitor PBO (Subramanyam *et al.*, 1989; Scott *et al.*, 1990a), indicative of mfo-conferred resistance.

1.4.3 GSTs

Increased GST activity has been reported to be involved in malathion resistance in *D. melanogaster* (Cochrane *et al.*, 1992), *M. domestica* (Motoyama and Dauterman, 1980; Shono, 1983), *Plutella xylostella* (Kao and Sun, 1991) and *Tribolium castaneum* (Reidy *et al.*, 1990). In general, the enzyme responsible for conferring resistance is found in both resistant and susceptible strains but is overexpressed in the resistant strain (Reidy *et al.*, 1990; Cochrane *et al.*, 1992). The increase in GST activity ranges from two- to 20-fold in *M. domestica* (Motoyama and Dauterman, 1980; Yeoh *et al.*, 1981; Bull and Pryor, 1990). In the resistant *D. melanogaster* strains studied by Cochrane *et al.* (1992), elevated levels of both classes of GSTs, GST1 and GST2, were found. Elevated

GST1 levels were caused by the overexpression of *DmGST1-1*, but mRNA levels of *DmGST2-1* were not elevated and no mechanism for the overproduction of the GST2 protein was proposed. In *P. xylostella*, GST activity is a minor contributor to malathion resistance, with the major resistance mechanism mediated by carboxylesterase activity (Kao and Sun, 1991). Increased GST activity does not appear to be a widespread mechanism of resistance to malathion.

1.4.4 Phosphatases

Malaoxon can be hydrolysed by phosphatase activity, like other OPs such as diazoxon (Section 1.3.2.3.1). Dimethyl phosphorothiolate and dimethyl phosphate are produced from malaoxon hydrolysis (Figure 1.8). Phosphatase products have been detected in assays for malathion hydrolysis in the mosquitoes *Anopheles albimanus* (Hemingway, 1985) and *Culex tarsalis* (Bigley and Plapp, 1962). In *Anopheles albimanus* the same rate of α– and β– naphthylacetate hydrolysis occurred in crude homogenates of both resistant and susceptible strains, thus this phosphatase activity appears unrelated to the "mutant aliesterase" hypothesis of Oppenoorth and van Asperen (1960). Bigley and Plapp (1962) found a high percent recovery of dimethyl phosphorothiolate and dimethyl phosphate from *C. tarsalis* homogenates in a malathion resistant strain. However, the ratio of phosphatase to carboxylesterase products was 1:11, suggesting that hydrolytic degradation of malathion by phosphatase activity was not the major mechanism of resistance. Further, there was little cross-resistance to paraoxon in the resistant strain, also implying a minor role of the phosphatase activity in resistance.

The seemingly minor role of phosphatases in malathion resistance, when they appear to play a much more important role in other OP resistance, may be explained by the properties of the phosphorothiolates (eg. malaoxon). Phosphatases tend to have very low turnover numbers and as phosphorothiolates bind more rapidly to AChE than phosphates, phosphatases may not degrade malaoxon fast enough to protect AChE from inhibition. In fact, all the above mentioned mechanisms appear to play relatively minor roles in malathion resistance. By far the most common mechanism of resistance is increased carboxylesterase activity.

1.4.5 Carboxylesterases

Many insect species are resistant to malathion via increased carboxylesterase activity (Table 1.5). Resistance via carboxylesterase activity can avoid the first rate limiting step in hydrolysis, the conversion of malathion to malaoxon, because it is able to hydrolyse the carboxylester linkages of both the phosphorodithioates and phosphorothiolates. Malathion carboxylesterase (MCE) hydrolyses the carboxylester linkages of malathion or malathion analogues and produces the more easily excreted α and β monocarboxylic acids. Occasionally both carboxylester linkages are hydrolysed to produce the diacid (Figure 1.8). Plapp and Eddy (1961) observed that TPP enhanced the toxicity of malathion in both resistant and susceptible strains of *M. domestica* and *C. tarsalis*. TPP reduces the production of α and β monocarboxylic acids and diacids and increases the formation of malaoxon by inhibiting carboxylesterase activity (Plapp *et al.*, 1963; Dyte and Rowlands, 1968), and it has been regarded as diagnostic for MCE-dependent detoxification in malathion resistant strains. Potentiation of malathion by TPP is consistent with it inhibiting the carboxylesterase detoxifying mechanism. Malathion resistance and hydrolysis is often also increased with the addition of PBO. PBO reduces

the mfo-dependent production of malaoxon, which can inhibit carboxylesterase activity (Kao *et al.*, 1985).

Most of the studies on MCE activity and malathion resistance in the species listed in Table 1.5 provide evidence for qualitative rather than quantitative changes in MCE activity between resistant and susceptible strains as the mechanism for malathion resistance. Studies of *M. domestica* suggest that the differences between the resistant and susceptible strains may be best explained by qualitative changes in MCE activity, but it was not clear in each study whether one or more enzymes were present in the resistant and susceptible enzyme preparations (Welling and Blaakmeer, 1971; Motoyama *et al.*, 1980; Picollo de Villar *et al.*, 1983; Kao *et al.*, 1985). The presence of more than one enzyme may obscure the individual effects of each enzyme to overall activity and resistance. Only one study has addressed the issue of qualitative versus quantitative changes in MCE activity in detail. The MAL-R strain of *C. tarsalis* has two MCE enzymes, one shared with the susceptible strain, and the second unique to the resistant strain (Whyard *et al.*, in press). The two MCE enzymes have different subcellular and tissue distributions and it is the presence of the second MCE that confers resistance (Whyard *et al.*, in press). Both MCE enzymes have been purified and their activities characterised kinetically. Although the K_M of the MCE unique to the resistant strain is five times higher than that of the MCE in the susceptible strain (12 versus 2.4 μM for malathion), the V_{max} is 18-fold higher than that of the susceptible MCE, indicating that the resistant MCE is capable of hydrolysing malathion significantly faster than the susceptible MCE (Whyard *et al.*, in press).

MCE activity is encoded by a monofactorial, autosomal gene in many species. These include the *Anopheles* species *arabiensis* (Lines *et al.*, 1984; Hemingway, 1985), *culicifacies* (Herath and Davidson, 1981a), and *stephensi* (Rathor and Toqir, 1981; Hemingway, 1982), the *Culex* species *tarsalis* (Matsumura and Brown, 1963) and *tritaeniorhynchus* (Takahashi and Yasutomi, 1987), *Laodelphax striatellus* (small brown plant hopper; Ozaki and Kassai, 1970), *M. domestica* (Shono, 1983), *Plodia interpunctella* (Indianmeal moth; Beeman and Schmidt, 1982) and *Tribolium castaneum* (White and Bell, 1988). The gene for MCE activity in *M. domestica* has been mapped to chromosome 2 (Shono, 1983). Interestingly, this is the chromosome where GST, mfo and aliesterase activities and the genes for diazinon and malathion resistance have also been mapped (Franco and Oppenoorth, 1962; Nguy and Busvine, 1960; Oppenoorth, 1967; Oppenoorth *et al.*, 1977). MCE activity has been mapped to chromosome 3R in *D. melanogaster* (Spackman *et al.*, 1994), the homologous chromosome to *M. domestica* chromosome 2 (Foster *et al.*, 1981; Weller and Foster, 1993), although MCE activity has not been reported to be involved in resistance to malathion in this species. MCE activity has also been mapped to chromosome 3 in *Anopheles arabiensis* and *stephensi* (Lines and Curtis, 1984; Rowland, 1985). These chromosomes are not homologous to chromosome 3R of *D. melanogaster* or chromosome 2 of *M. domestica*.

The original "mutant aliesterase" hypothesis was also formulated to incorporate malathion resistance (Oppenoorth and van Asperen, 1960). However, there appears to be little evidence for a correlation between aliesterase activity as measured by α-naphthylacetate or methyl butyrate hydrolysis and malathion resistance conferred by increased MCE activity. Some species do show decreased aliesterase and increased carboxylesterase activities: *Chrysomya putoria* (Townsend and Busvine, 1969), *Plodia interpunctella* (Beeman and Schmidt, 1982) and *Culex tarsalis* (Whyard *et al.*, in press). However, there are other species with equal rates of α-naphthylacetate hydrolysis in

resistant and susceptible strains. Interestingly, one of these species is that for which the mutant aliesterase hypothesis was originally formulated, *M. domestica*. The Hirokawa strain possesses high malathion carboxylesterase activity without any change in activity to α-naphthylacetate (Kao *et al.*, 1984; Kao *et al.*, 1985; Motoyama *et al.*, 1980; Picollo de Villar *et al.*, 1983). Other species with equal α-naphthylacetate hydrolysis in resistant and susceptible strains are *Anopheles arabiensis* (Hemingway, 1985) and *Anopheles albimanus* (Hemingway *et al.*, 1985). Therefore, malathion resistance conferred by increased carboxylesterase activity does not fit the mutant aliesterase hypothesis in all species.

Table 1.5 Examples of species with increased carboxylesterase activity as a malathion resistance mechanism.

Species	Reference
Coleoptera:	
Tribolium castaneum	Dyte and Rowlands, 1968; White and Bell, 1988; Subramanyam *et al.*, 1989
Diptera:	
Anopheles albimanus	Hemingway *et al.*, 1985
Anopheles arabiensis	Hemingway, 1985
Anopheles culicifacies	Herath *et al.*, 1987; Malcolm and Boddington, 1989
Anopheles stephensi	Herath and Davidson, 1981b; Hemingway, 1982; Scott and Georghiou, 1986; Rowland and Hemingway, 1987
Ceratitis capitata	Koren *et al.*, 1984
Chryosmya putoria	Townsend and Busvine, 1969
Culex quinquefaciatus	Miyata *et al.*, 1984a; Miyata *et al.*, 1984b
Culex tarsalis	Plapp and Eddy, 1961; Bigley and Plapp, 1962; Matsumura and Brown, 1963; Plapp *et al.*, 1963; Plapp and Tong, 1966; Ziegler *et al.*, 1987; Whyard *et al.*, 1994a; Whyard *et al.*, in press
Musca domestica	Plapp and Eddy, 1961; Plapp *et al.*, 1963; Plapp and Tong, 1966; Welling and Blaakmeer, 1971; Welling *et al.*, 1973; Motoyama *et al.*, 1980; Yeoh *et al.*, 1981; Picollo de Villar *et al.*, 1983; Shono, 1983; Kao *et al.*, 1984; Kao *et al.*, 1985
Homoptera:	
Laodelphax striatellus	Sakata and Miyata, 1994
Myzus nicotianae	Abdel-Aal *et al.*, 1990; Abdel-Aal *et al.*, 1992
Nilaparvata lugens	Chen and Sun, 1994
Heteroptera:	
Nepthottettix cincticeps	Miyata and Saito, 1976
Lepidoptera:	
Plodia interpunctella	Beeman and Schmidt, 1982; Halliday, 1988

Table 1.6 Summary of known OP resistance genes and mechanisms in *Musca domestica*, *Drosophila melanogaster* and *Lucilia cuprina*.

Gene	Biochemical Phenotype	Resistance Mechanism	Insecticides	Chromosome Location			References
				Musca domestica	*Drosophila melanogaster*	*Lucilia cuprina*	
pen[a]	Reduced penetration	Increased cuticular phospholipid concentration	Resistance to most classes of insecticides	3			Hoyer and Plapp, 1968; Plapp and Hoyer, 1968
Ace	Acetylcholinesterase (AChE)	Altered AChE with reduced binding affinity	Carbamates and OPs	2	3R		Oppenoorth, 1979; Plapp, 1976; Morton and Singh, 1982
Ox_2	Mixed function oxidase activity	Increased or decreased activity	Carbamates, DDT, OPs and pyrethroids	2	3R		Tsukamoto and Sawicki, 1966; Tsukamoto *et al.*, 1968; Plapp and Casida, 1969; Hällström, 1985; Hällström and Blanck, 1985; Houpt *et al.*, 1988
DDTmd	Mixed function oxidase activity	Increased activity	Carbamates, DDT, OPs and pyrethroids	5	2R	6 (*Rop2*)?[b]	Sawicki and Farnham, 1967; Plapp and Casida, 1969; Arnold and Whitten, 1976; Hällström, 1985; Hällström and Blanck, 1985; Houpt *et al.*, 1988

Table 1.6 continued

g	Glutathione *S*-transferase	Increased activity	OPs	2 (*MdGST1*)	3R (*DmGST1*)	4 (*LcGST1*)?	Oppenoorth *et al.*, 1977; Ottea and Plapp, 1984; Parkes *et al.*, 1993; Board *et al.*, 1994
GST2	Glutathione *S*-transferase	Increased activity	OPs		2R (*DmGST2*)		Beall *et al.*, 1992
a	Aliesterase activity	Decreased activity	OPs	2	3R (*Ali*)	4 (*Rop1*)?	Franco and Oppenoorth, 1962; Arnold and Whitten, 1976; Wang and Plapp, 1980; Hughes and Devonshire, 1982; Spackman *et al.*, 1994
MCE	Malathion Carboxylesterase	Increased activity	Malathion	2	3R (*Mce*)	4 (*Rmal*)	Shono, 1983; Hughes *et al.*, 1984; Raftos and Hughes, 1986; Spackman *et al.*, 1994

[a]The gene name is that for the *M. domestica* gene. Where the name differs for the other two species it has been included in parentheses under that species's column.

[b]The question mark indicates that preliminary evidence suggests that these *L. cuprina* genes are homologous to the *M. domestica* and *D. melanogaster* genes.

1.5 OP Resistance in *Lucilia cuprina*

There appears to be a limited number of mechanisms by which an insect can develop resistance to OPs. Most studies of OP resistance have concentrated on *M. domestica*. Chromosome 2 in *M. domestica* is a major linkage group controlling metabolic resistance and is homologous to *D. melanogaster* chromosome 3R and *Lucilia cuprina* chromosome 4 (Foster *et al.*, 1981; Weller and Foster, 1993). Aliesterase, GST, MCE and mfo activities have all been mapped to chromosomes 2 in *M. domestica* and 3R in *D. melanogaster* and MCE activity to chromosome 4 in *L. cuprina* (Table 1.6). The lack of three point test crosses and the occurrence of inversions on chromosome 2 of *M. domestica* (Hiroyoshi, 1977; Wang and Plapp, 1980) have led to varying distances being reported between resistance genes and the mutants with which they have been mapped. However, more consistent data are available for both *D. melanogaster* and *L. cuprina*. *L. cuprina* is a useful model organism to study biochemistry and genetics of insecticide resistance as it can easily be reared in the laboratory, has a relatively short generation time of three weeks, and although few resistance genes have been mapped in *L. cuprina*, the genetics have been well characterised.

L. cuprina has become a major pest to both the wool and meat industries in Australia and New Zealand due to its ability to produce cutaneous myiasis, or flystrike, and its resistance to insecticidal control. Initially, arsenicals were used to control populations of *L. cuprina*, but were replaced by dichlorodiphenyl trichloroethane (DDT) (Shanahan and Roxburgh, 1974). The simpler application and handling of the cyclodienes, dieldrin and aldrin, led to the replacement of arsenicals and DDT in 1955, although no resistance to these insecticides had developed. However, resistance to dieldrin developed rapidly, within three years, and OPs replaced dieldrin in 1957 (Shanahan, 1958; Shanahan and Roxburgh, 1974; Shanahan and Hughes, 1979). The mechanisms of resistance to arscenicals, DDT and cyclodienes are different from those to OPs. DDT resistance is primarily conferred by altered sodium channels (Taylor *et al.*, 1993; Williamson *et al.*, 1993; Knipple *et al.*, 1994), but also by dehydrochlorinase actvity (a GST; Tsukamoto and Suzuki, 1964; Clark and Shamaan, 1984), whereas cyclodiene resistance is conferred by an altered GABA-gated chloride channel (ffrench-Constant, 1994; ffrench-Constant *et al.*, 1993).

The use of diazinon, the primary control OP, continued after resistance was established (Shanahan, 1966), probably due to the lack of an alternative insecticide (Hughes and McKenzie, 1987). OPs still comprise a large part of the control strategy for *L. cuprina* (G. Levot, personal communication), despite the high frequency of diazinon resistant flies in field populations (97% in 1980; Hughes, 1981) and 98.1% in 1992 (G. Levot, personal communication) and cross resistance to other OPs (O'Flynn and Green, 1980; Hughes, 1981; Levot, 1990). Diazinon remains in use today as it can confer about six weeks protection from fly strike (Levot, 1990).

Cyromazine (2-cyclopropylamino-4,6-diamino-S-triazine) has been a major insecticide used for control since 1978 and resistance has not yet been detected in the field (Hughes and McKenzie, 1987; G. Levot, personal communication). The mode of action of cyromazine is not known but larvae of *Ceratitis capitata* (Viñuela and Budia, 1994), *L. cuprina* (Binnington *et al.*, 1987) and *Manduca sexta* (tobacco budworm; Kotze and Reynolds, 1990; Kotze and Reynolds, 1991) treated with cyromazine have shown severe cuticle abnormalities, indicating that cyromazine effects the pathway of cuticular formation. Cyromazine poisoning is associated with a marked increase in internal pressure and reduced extensibility of the cuticle (Reynolds and Blakey, 1989) that

eventually leads to lesions in the bodywall from which body fluids are lost, ultimately resulting in death (Kotze and Reynolds, 1990).

1.5.1 General OP Resistance and Esterase E3 Activity

Two genes appear to be involved in general OP resistance (ie. not malathion-specific resistance), *Rop1* and *Rop2*. *Rop1* is located on chromosome 4, 4.4 map units from the visible marker *bubbled wings* and 15.2 from *radial wing veins* (Weller and Foster, 1993). Three alleles of the *Rop1* locus have been described, *Rop1A*, *Rop1B* and *Rop1C* (Arnold and Whitten, 1976). *Rop1A* and *Rop1B* have both been found in field collections of resistant flies. *Rop1A* appears to be the most frequent in field populations and confers a higher level of resistance in adults than *Rop1B*. However, the converse is true for larval resistance; *Rop1B* confers higher resistance than *Rop1A* and larval resistance for both alleles is higher than adult resistance (12.5- and 5-fold respectively; Arnold and Whitten, 1976). The *Rop1C* allele resulted from laboratory selection experiments and has not been detected in field populations. It confers even higher resistance in both adults and larvae, with larval resistance 15.6-fold higher than adult (Arnold and Whitten, 1976). *Rop2*, is located on chromosome 6, 10.2 map units from *deformed wings* (Foster *et al.*, 1981). Only one resistance allele of *Rop2* has been described and the level of resistance conferred is less that that of the *Rop1* alleles, with adult resistance about 2-fold higher than larval resistance (Arnold and Whitten, 1976). Both *Rop1* and *Rop2* are incompletely dominant genes and their intergenic interaction is multiplicative (Arnold and Whitten, 1976), indicating that their biochemical phenotypes are different (Plapp, 1970).

Early experiments on the biochemical basis of general OP resistance showed that PBO, an inhibitor of mfos (Wilkinson, 1971) and TBTP, an esterase inhibitor (Plapp and Tong, 1966) increased resistance in both susceptible and resistant strains, implying that both mfo and esterase activities are involved in resistance (Attia *et al.*, 1979; Hughes, 1982). While PBO synergised resistance in the susceptible strain more than the resistant strain, TBPT had the converse effect, suggesting that esterase activity played a more important role in resistance than mfo activity (Hughes, 1982). This was further supported by the characterisation of parathion and paraoxon metabolism *in vivo* and *in vitro* in resistant and susceptible flies by Hughes and Devonshire (1982). Their study showed that there was no apparent difference between susceptible and resistant flies in either the penetration of parathion or paraoxon nor in the sensitivity of AChE to inhibition by these OPs. Eight metabolites were produced from the degradation of parathion *in vivo* but only three were produced in measurable quantities: paraoxon, diethyl phosphorothioate (DEPTA) and diethyl phosphate (DEPA) (Hughes and Devonshire, 1982). Paraoxon is the desulphurated or active form of parathion (Eto, 1974). The production of paraoxon implies the presence of an mfo activity able to convert parathion to paraoxon, and DEPA is a hydrolysis product of paraoxon. DETPA, on the other hand is a direct product of parathion hydrolysis by mfo activity (Eto, 1974). The production of DEPA, following the conversion of parathion to paraoxon, did not require the addition of co-factors. Hughes and Devonshire (1982) concluded that resistance was primarily caused by the presence of a microsomal esterase able to degrade paraoxon more rapidly in resistant flies and this esteratic activity corresponded to that observed by Hughes (1982) and mapped to chromosome 4.

Increased mfo activity was presumed to be responsible for the production of DETPA because it required NADPH. The addition of NADPH also caused a minor

increase in the production of DEPA (Hughes and Devonshire, 1982). Mfos have been implicated in OP resistance of *L. cuprina* larvae (Terras *et al.*, 1983) and laboratory pyrethroid-selected strains that exhibit cross-resistance to diazinon have higher levels of mfo activity than susceptible strains, indicating that mfos can be important in resistance to diazinon (Kotze, 1993). To explain why resistance mapped to chromosome 4, yet there appeared to be two mechanisms involved, Hughes and Devonshire (1982) suggested that either changes in regulation affected the genes encoding both activities or that they were tightly linked on chromosome 4. The hypothesis of two tightly linked genes is supported by the finding of Arnold and Whitten (1976) that *Rop1A* and *Rop1B* could be two tightly-linked genes rather than alleles of *Rop1*. *Rop1A* could encode the esterase activity and *Rop1B*, the mfo activity. This situation is consistent with the higher level of resistance conferred by *Rop1A*. On the other hand, the mfo activity could be encoded by *Rop2*. Mfo activities have been mapped to genes on chromosome 5 in *M.domestica* (Oppenoorth, 1967; Plapp and Casida, 1969; Plapp *et al.*, 1976; Hodgson *et al.*, 1993) and 2R in *D. melanogaster* (Hällström, 1985; Hällström and Blanck, 1985; Houpt *et al.*, 1988; Waters and Nix, 1988), homologous chromosomes to *L. cuprina* chromosome 6 where *Rop2* maps (Foster *et al.*, 1981; Weller and Foster, 1993). However, as the mfo activity has not been mapped, there is no direct evidence for either of these hypotheses.

Sixteen esterase isozymes have been identified from *L. cuprina* after separation by native polyacrylamide gel electrophoresis (PAGE) and detection with α- and β-naphthylacetate as substrates (Hughes and Raftos, 1985). One of these, esterase E3, was found to be associated with OP resistance. The E3 isozyme was found in susceptible homozygotes and resistant heterozygotes but was absent from homozygous resistant flies. Thus, resistant flies appeared to be null for E3. Alternatively, they might carry a non-staining form of E3 that Hughes and Raftos (1985) termed "E3 null". The E3 null phenotype was mapped to the same region of chromosome 4 as *Rop1* and analysis of the frequencies of E3 null (0.97) and diazinon resistance (0.967) in field populations suggested that E3 null may be a resistance conferring allele of *Rop1* (Hughes and Raftos, 1985). However, in the mapping experiments of Hughes and Raftos (1985), three of the 64 backcross progeny expected to be homozygous for resistance on the basis of discriminating doses expressed the E3 staining phenotype. There are two possible explanations for this observation. Firstly, the E3 gene and *Rop1* are not allelic but linked on chromosome 4. If they are two independent genes, the recombination frequency would place them 4.7±2.6 map units apart. On the other hand, McKenzie *et al.* (1982) found that the dose response lines for the three diazinon resistant genotypes (ie. +/+; R/+; R/R) overlapped and they recorded approximately seven percent incorrect assignment of genotype to phenotype. The expected number of incorrect assignments based on this assumption is 4.5 from the 64 backcross progeny. Therefore the three E3 staining individuals could have been resistant heterozygotes that escaped the screening procedure.

The association between E3 null and OP resistance led Hughes and Raftos (1985) to propose that E3 null was a mutant aliesterase similar to that proposed for OP resistant *M. domestica* (Section 1.3.2.3.3). The *M. domestica* aliesterase appears to have mutated to confer resistance via degradation of OPs, and simultaneously lost the ability to hydrolyse artificial substrates such as α- and β-naphthylacetate. The E3 staining enzyme has been characterised as a carboxylesterase (or a B-esterase, Section 1.3.2.3) on the basis of its substrate specificity and response to various esterase inhibitors (Parker *et al.*, 1991). Therefore, if the E3 null esterase is responsible for the phosphatase activity observed by Hughes and Devonshire (1982), the resistance mutation must cause a change

from the E3 staining carboxylesterase activity to the E3 null phosphatase activity. The E3 null phenotype will be referred to as "E3 non-staining" on the assumption that an esterase (phosphatase) is present corresponding to E3 staining rather than a true null, where the lack of staining would represent the absence of the esterase.

The E3 staining enzyme has been partially purified and is a 70kDa monomeric protein with a K_M for α-naphthylacetate of 50±16μM and V_{max} of 7±1 μmol/min/mg (A. Parker, personal communication). The enzyme present in resistant strains cannot be detected after native PAGE with diverse ester substrates and has not been purified. The gene encoding the staining form of E3 has recently been cloned and the protein expressed using a baculovirus expression system (R. Newcomb, personal communication). Attempts to express the non-staining allele are underway, as is the development of a radiometric assay to determine if the E3 non-staining enzyme can hydrolyse OPs (P. Campbell, personal communication). To date there is no direct evidence to show that the E3 non-staining enzyme is encoded by an allele of *Rop1*, that it is directly involved in OP resistance, or that it is capable of hydrolysing OPs. It is possible that the gene encoding E3 is in gametic disequilibrium with *Rop1* and recombination between the non-staining and resistance alleles at these loci is extremely rare, not detectable in the sample sizes that have been examined. The most conclusive piece of evidence that E3 non-staining is causally related to OP resistance relies on four ethyl methanesulphonate (EMS) induced diazinon resistant mutants that map to the *Rop1* locus and also express the E3 non-staining phenotype (McKenzie *et al.*, 1992). In addition to the data outlined above, evidence from the EMS mutants suggests that the E3 non-staining enzyme is an allele of *Rop1* and confers resistance by hydrolysing phosphate esters of OPs.

1.5.2 Malathion Resistance and MCE Activity

Malathion has not been registered for use against *L. cuprina*. Trials conducted in 1955 showed that 0.1% and 0.25% malathion could afford protection from flystrike for approximately seven weeks before it lost efficacy (Riches and O'Sullivan, 1957). However, diazinon could give 23 weeks protection at 0.008% and was preferential to malathion (Riches and O'Sullivan, 1957). Malathion is registered for use in a mixture of OPs for control of sheep body lice (*Damalinia ovis*), sheep ked (*Melophagus ovinus*) and sheep itch mites (*Psorergates ovis*) (Hughes and McKenzie, 1987). In 1984, Hughes *et al.* recorded 11% resistance to malathion in field populations, which seemed surprising given that malathion had not been used to control *L. cuprina*. However, both adults and larvae may come into contact with malathion after sheep have been treated for body lice, ked or itch mite and therefore resistance could have been selected indirectly.

The genetic basis of malathion resistance is a single dominant gene, *Rmal*, located on chromosome 4, 24.5±1.3 map units from the *singed vibrissae* marker (Hughes *et al.*, 1984; Raftos and Hughes, 1986). The mapping of *Rmal* places it close to *Rop1* (23.3±2.3 map units from the *singed vibrissae* maker; Raftos and Hughes, 1986), the locus encoding general OP resistance. Although the two genes were not be distinguished by these mapping experiments, allelism tests showed that they were independent but tightly linked (Raftos and Hughes, 1986). No recombination was found between *Rmal* and a single locus controlling MCE activity. Thus, unlike the situation for E3 and *Rop1*, there is direct evidence that *Rmal* encodes MCE activity (Hughes *et al.*, 1984; Raftos and Hughes, 1986). Raftos and Hughes (1986) suggested that, as various esterases were clustered on chromosome 3R in *D. melanogaster* and chromosome 2 in *M. domestica*, and since these chromosomes have been shown to be homologous to chromosome 4 in *L.*

cuprina (Foster *et al.*, 1981; Weller and Foster, 1993), *Rop1* and *Rmal* may also belong to an esterase cluster.

There were no differences found in the rates of penetration, excretion, storage or sensitivity of AChE for either malathion or malaoxon between a malathion resistant strain, RM, and the LS susceptible strain (Raftos, 1986). The addition of the co-factors NADPH and GSH did not increase the production of malathion metabolites, nor did PBO inhibit their production (Raftos, 1986). Thus, mfo and GST activities do not appear to be involved in malathion resistance in *L. cuprina*. However, the RM strain had 1.5- to 3.6-fold more MCE activity compared to LS (Hughes *et al.*, 1984) and RM hydrolysed malathion more rapidly to α and β malathion monocarboxylic acids *in vivo* and *in vitro* than LS, suggesting that resistance is derived by enhanced esteratic activity. Furthermore, MCE activity and malathion resistance were completely suppressed by the carboxylesterase inhibitor TPP (Hughes *et al.*, 1984; Raftos, 1986). The increased MCE activity conferred cross-resistance only to malathion analogues such as malaoxon (>100-fold, P. B. Hughes unpublished data, reported in Hughes *et al.*, 1984) and phenthoate (118-fold, (Hughes *et al.*, 1984), which is similar to other characterised MCE activities (Townsend and Busvine, 1969; Beeman and Schmidt, 1982).

Enhanced degradation of malathion occurred in both mitochondria and microsomes and preliminary kinetic studies on crude homogenates found that the K_M of the mitochondrial enzyme was the same for both LS and RM (Raftos, 1986). Raftos (1986) suggested that the increased degradation of malathion in RM was due to increased production of an MCE found in both resistant and susceptible strains. However, the K_M of the microsomal fraction enzyme differed between RM and LS and similar K_Ms do not necessarily mean that resistance is conferred by increased production of the same MCE. The K_M of the RM enzyme was higher than that of LS indicating that the increased degradation in microsomes was due to a different enzyme (Raftos, 1986), and it may be this enzyme that confers resistance. A second study on the subcellular localisation of MCE activity in RM found that most MCE activity is in the cytosol (~50%) with approximately 26% localised to the mitochondria (Whyard *et al.*, 1994b). A similar subcellular distribution was found for both RM and another susceptible strain, der-S (Whyard *et al.*, 1994b).

An MCE has been purified from the RM strain (Whyard *et al.*, 1994b). It is a 60.5KDa monomer with a K_M for malathion of 11.0±0.4μM and a V_{max} of 775±28 nmol malathion/min/mg (Whyard *et al.*, 1994b). Titration of MCE activity with DFP revealed a single catalytic site and characterised the MCE as a B esterase (Whyard *et al.*, 1994b). In a separate study, Whyard and Walker (1994) reported the purification of MCE from another strain, der-L, which is 11 times less resistant to malathion than RM and has ten times less MCE activity. The purified der-L MCE was kinetically identical to that found in RM, but nine times less abundant than RM, suggesting that the greater resistance of RM was due to more of the same protein found in both strains.

Two pieces of indirect evidence suggest that the difference in MCE activity between resistant and susceptible *L. cuprina* may be qualitative rather than quantitative. Firstly, the high k_{cat}/K_M ratio of the RM MCE indicates that it can hydrolyse malathion efficiently. It also has a high turnover rate of malathion (k_{cat}=46 min^{-1}) relative to the aphid esterase E4 (Section 1.3.2.3.2). E4 confers resistance by sequestration and limited hydrolytic activity and has a turnover number for paraoxon (0.01min^{-1}) 4500 times lower than that of MCE for malathion. It also accounts for 3% of the total soluble protein, which is 64 times that for MCE (0.05% total soluble protein; Whyard *et al.*, 1994b).

Secondly, there are two MCE enzymes found in *C. tarsalis*, one common to both resistant and susceptible mosquitos and a second unique to the resistant strain (Whyard *et al.*, in press). Resistance is thus conferred by a qualitative change in MCE activity. The enzyme unique to the resistant strain is 2.8 times slower and less efficient than the *L. cuprina* MCE enzyme and accounts for only 0.02% of the total extractable protein, a similar amount to the *L. cuprina* MCE. These comparisons suggest that there may be a qualitative difference in MCE between malathion susceptible and resistant *L. cuprina*.

1.5.3 Propetamphos Resistance

Propetamphos was introduced as a control agent for *L. cuprina* and the sheep body louse and ked in 1986 (Hughes and McKenzie, 1987). Resistance has not yet been detected in field populations of *L. cuprina*, but has been generated in laboratory selection experiments (Levot, 1990). Propetamphos contains both types of ester bond, phosphoryl and carboxyl, and an amide bond (Table 1.1). It may be hydrolysed at any one of these bonds, by phosphatase, carboxylesterase, amidase, GST or mfo activities. Therefore, it may be hydrolysed by either the E3 phosphatase activity at the phosphoester bond, after desulphuration by mfos, or by MCE activity at the carboxylester bond, generating more soluble and easily excreted products.

1.6 Aims of This Study

The aim of my study was to understand the biochemical and genetic basis of OP resistance in *L. cuprina*, with particular reference to malathion resistance. I formulated my aims into three broad objectives.

The first objective was to elucidate the genetic basis of MCE activity and malathion resistance. This objective was divided into two parts. The first was to map MCE activity and, in particular, to determine whether MCE activity was clustered with other esterase genes on chromosome 4, as implicated by Raftos (1986). Both malathion and diazinon resistances had been mapped with respect to a visible marker approximately 20 map units away, but had not been mapped with respect to each other. Initially I characterised the genetics of the biochemical phenotypes of the two resistance genes, *Rop1* and *Rmal*, by mapping MCE activity with respect to E3 activity. In addition, E4 was included in the study because Lai-Fook and Smith (1992) mapped this esterase to within 16 map units of the *singed vibrissae* marker on chromosome 4 and it could therefore map close to *Rop1* and *Rmal*. Furthermore, E9 has been implicated in OP resistance because staining and non-staining isozymes also appear to segregate with OP susceptible and resistant strains, and it has similar spatial and temporal distribution to E3 (Parker *et al.*, 1991), but there was no preliminary data on the map position of this esterase.

The second part of the first objective was to investigate the resistance status of the strains used in the mapping experiments. The characterisation of MCE activities from the mapping strains revealed three distinct MCE phenotypes: high, intermediate and low. It was therefore important to test the resistance status of representative strains from each of the three MCE phenotypes. The results of the mapping and resistance experiments are outlined and discussed in Chapter 2. Briefly, MCE, E3 and E9 activities all cluster on the left of the *bubbled wing* (*bu*) marker, with E4 four map units on the other side of *bu*. The strains with low and intermediate MCE activities are equally susceptible to malathion, with only high MCE activity conferring malathion resistance.

The second objective of my research was to characterise the biochemistry and physiology of MCE activity in strains representative of the high, intermediate and low phenotypes. Previous studies had characterised the subcellular distribution, pH optima and response to two inhibitors of MCE activity. However, a detailed study of the tissue distribution, developmental profile or response to various esterase inhibitors had not been performed for MCE activity in either resistant or susceptible strains. Chapter 3 describes the approach taken to characterise the biochemistry and physiology of MCE activity in strains representative of the high, intermediate and low phenotypes. The results indicate that the MCE in resistant strains is different from those found in the susceptible low and intermediate strains.

The third objective was to investigate an apparent negative association between MCE activity and E3 activity and its relevance to resistance. The characterisation of the MCE activities from the mapping strains not only revealed three MCE phenotypes but also showed that high and intermediate MCE activities were associated with E3 staining activity and low MCE activity with E3 non-staining activity. I was interested to determine whether the negative association was an artifact of the small number of strains sampled or a more general phenomenon. To address this question, lines were made homozygous for chromosome 4, both with and without screening for malathion resistance. These lines and a sample of five mass populations were tested for MCE and E3 activities and resistance to malathion, diazinon and propetamphos. The results of these experiments are described in Chapter 4 and they show that there is a negative association between malathion and diazinon resistances that extends from the iso-chromosome lines to the field strains.

Chapter 5 draws together the conclusions of the three objectives and discusses these with reference to the literature and preliminary molecular genetic data generated by other researchers since the completion of the experimental work documented here.

CHAPTER 2

MALATHION RESISTANCE, MCE ACTIVITY AND GENETIC MAPPING

2.1 Introduction

Chapter 1 has described mutations in at least two carboxylesterase genes that appear to confer OP resistance in the sheep blow fly, *Lucilia cuprina* (Hughes *et al.*, 1984; Raftos and Hughes, 1986). Two resistance genes, *Rop1* and *Rmal*, are closely-linked. *Rop1* is 21.8±1.3 and *Rmal* is 24.5±1.3 map units proximal to the *singed vibrissae* (*sv*) marker on chromosome 4 (Raftos and Hughes, 1986). The OP susceptible allele of *Rop1* is thought to encode the carboxylesterase E3 (Arnold and Whitten, 1976; Hughes and Raftos, 1985; Parker *et al.*, 1991; McKenzie *et al.*, 1992). The mutation at this locus that confers resistance to a broad range of OPs via phosphatase activity (Hughes, 1982) is also associated with the apparent loss of the E3 allozyme (Hughes and Raftos, 1985; Section 1.5.1). Similar findings have been reported for some OP resistant *M. domestica* strains, where the acquisition of phosphatase activity is associated with reduced carboxylesterase (aliesterase) activity (Oppenoorth and van Asperen, 1960). Both sets of data have been interpreted in terms of the so-called 'mutant aliesterase' hypothesis. This hypothesis proposes that the simultaneous loss of carboxylesterase activity and gain of phosphatase activity are due to a structural mutation in a single gene affecting the substrate specificity of the enzyme. The loss of carboxylesterase activity renders the enzyme unable to hydrolyse artificial substrates like methylbutyrate or α-naphthylacetate, and the gain of phosphatase activity enhances the ability to degrade OPs (van Asperen and Oppenoorth, 1959; Oppenoorth and van Asperen, 1960; Section 1.3.2.3.3).

The second resistance gene, *Rmal*, only confers resistance to malathion and other related OPs with carboxylester bonds. It is associated with elevated MCE activity *in vitro* and enhanced malathion degradation *in vivo* (Hughes *et al.*, 1984; Raftos, 1986; Raftos and Hughes, 1986; Section 1.5.2). Two other esterases are also relevant to the genetics of OP resistance in *L. cuprina*. One of these, E9, is similar to E3 in its spatial and temporal distribution and response to esterase inhibitors, and like E3, it also shows a non-staining phenotype in some strains resistant to general OPs (Parker *et al.*, 1991). The genetics of E9 have not been reported previously. The other enzyme, the haemolymph esterase E4, has no known biochemical or physiological association with OP resistance, but preliminary genetic data map it 16.3 map units proximal to *sv* (Lai-Fook and Smith, 1992), which would place it in close vicinity to *Rop1* and *Rmal*.

This chapter describes the characterisation of MCE specific activities among seven strains of *L. cuprina* and further genetic analyses of the MCE, E3, E4 and E9 enzymes. There is a third MCE phenotype intermediate in activity between the previously reported high and low phenotypes. All three MCE phenotypes and the esterases E3 and E9 are shown to map within approximately one map unit of each other, with E4 six map units away. The MCE/E3/E9 cluster is apparently homologous to one recently identified in *D. melanogaster* (Spackman *et al.*, 1994).

2.2 Materials and Methods

2.2.1 Strains

RM2-6 (malathion susceptible) and RM8 (malathion resistant) are iso-chromosome 4 lines derived from the RM strain (malathion resistant; Raftos and Hughes, 1986) as previously described (Whyard *et al.*, 1994b). RM8 is the same line as the der-R line of Whyard *et al.* (1994b). The der-S strain of Whyard *et al.* (1994b) was also isolated from RM and has since been lost, however RM2-6 is similar to der-S in its MCE specific activities and LD_{50} values for malathion.

Five other strains were used in various genetic analyses described below. Two of these, LS2 and Q4, are also iso-chromosome 4 lines. LS2 was derived from a field strain susceptible to a wide range of OPs, including malathion, while Q4 is a field-derived line resistant to general OPs (Parker *et al.*, 1991). The Dua-91 strain was collected from Cowra, NSW and is resistant to general OPs. The *sv bu ra* marker strain was constructed in the laboratory and is homozygous for three recessive markers on chromosome 4: *sv* (*singed vibrissae*), *bu* (*bubble wings*), and *ra* (*radial vein gaps*). The second laboratory strain, *Sh*, carries the dominant marker *Short Bristles*. The *Sh* mutation is also homozygous lethal and is maintained as a heterozygote over a wild-type allele. The markers have been previously documented by Maddern *et al.* (1986) and Weller and Foster (1993).

Larvae were raised on a combination of sheep liver and meat meal media at 27°C (Foster *et al.*, 1981). Adult flies were maintained on a diet of sugar and water and given a protein feed prior to oviposition. Flies for all experiments were used within the first week of eclosion.

2.2.2 Malathion Carboxylesterase Assay

MCE activity was assayed using the partition method of Ziegler *et al.* (1987) as modified by Whyard *et al.* (1994b). Except where indicated, individual flies were homogenised in 1ml MCE homogenisation buffer (10mM imidazole-HCl, pH7.0, containing 1% Triton X-100 (v/v), 1mM phenylthiourea (PTU) and 1mM dithiothreitol (DTT)). Homogenates were then clarified by centrifugation at 12000g for ten minutes at 4°C. Samples of 15µl (for high and intermediate strains) or 75µl (for low strains) of supernatant were added to 75µl dilution buffer (10mM imidazole-HCl, pH7.0) containing [^{14}C]-malathion (Amersham; 103 mCi/mmole labelled at both the methylene carbons of the succinate moiety) at a final concentration of 280nCi. The malathion concentration was adjusted to 37.5µM by the addition of unlabelled malathion (99%; Riedel-de-Haën Ag., Seelze, Germany) and where necessary, the final volume was brought to 150µl with dilution buffer. The assay mixture was incubated at 25°C for one hour, then 300µl of dilution buffer was added and the undegraded malathion extracted three times with 970µl of chloroform. The concentration of carboxylic acids of malathion in 300µl of aqueous phase was determined using a Beckman 2800 liquid scintillation counter. Each homogenate was assayed in duplicate. Homogenates were then stored at -20°C before their protein concentrations were determined (method of Bradford (1976) with ovalbumin as the standard). Specific activity is expressed as pmol malathion hydrolysed per minute per mg protein. The non-enzymatic degradation of malathion was corrected for by subtracting the activity of negative controls, in which homogenates had been heated at 100°C for ten minutes prior to the assay.

2.2.3 Malathion Resistance Assay

Dose-mortality relationships were determined for RM8, LS2 and RM2-6 by topically applying 1µl of increasing concentrations of malathion in acetone to the upper thorax of fifty adult flies of each sex, three to five days after eclosion. Control flies were tested with acetone and mortality was recorded after 24 hours. The malathion concentration at which fifty percent of the flies died was estimated using probit analysis (Finney, 1971) as implemented in SAS, version 6.03 (SAS® Technical Report P-179, 1988). The resistance status of the four other strains was determined using a discriminating dose of 2µg/µl malathion (Hughes *et al.*, 1984).

2.2.4 Native PAGE Electrophoresis

Esterases E3, E4 and E9 were separated by discontinuous native polyacrylamide gel electrophoresis (PAGE) using the method of Hughes and Raftos (1985) and detected with the histochemical substrates α- and β-naphthylacetate. E3 and E9 activities were scored using 10µl of whole fly homogenate prepared by homogenising individual flies in 600µl of phosphate homogenisation buffer (0.01M phosphate buffer, pH6.8 containing 20% (w/v) sucrose, 1mM EDTA and 0.5% (v/v) Triton X-100; Parker *et al.*, 1991). Extracts also assayed for MCE activity were prepared in essentially the same way using MCE homogenisation buffer in place of phosphate homogenisation buffer. E4 activity was scored using 2 to 3µl of haemolymph, diluted to 7µl with phosphate homogenisation buffer.

2.2.5 Genetic Mapping

2.2.5.1 *High/Low MCE and E3 Activities*

The aims of this cross were first to confirm that the difference between high and low MCE specific activities behaved as a single gene and second to map the MCE activities with respect to the E3 staining versus non-staining phenotypes. Virgin Q4 females were crossed with RM8 males and the F_1 males were individually assayed for MCE and E3 activities, while the F_1 females were backcrossed to Q4 males. (There was no difference in activity between male and female F_1 progeny; results not shown). The backcross progeny were assayed individually for MCE and E3 activities.

2.2.5.2 *High/Intermediate MCE Activities and sv bu ra*

Crosses were constructed to map a third MCE phenotype observed during the course of this work. This phenotype is characterised by a rate of malathion hydrolysis intermediate between low and high MCE specific activities. The *sv bu ra* marker strain displays this intermediate MCE activity and was used to test whether the high versus intermediate activity difference behaved as a single gene and to map it with respect to three visible markers on chromosome 4. Eighteen *sv bu ra* females were crossed individually to RM8 males. Three F_1 males from each of the single female lines were assayed individually for MCE activity and the females were backcrossed to *sv bu ra* males. (Again, there was no difference in activity between male and female F_1 progeny; results not shown). The backcross progeny were scored for the visible markers and then assayed for MCE activity.

2.2.5.3 *Intermediate/Low MCE and E3 Activities*

A cross between LS2 females and RM2-6 males was established to test whether the difference in activity between low and intermediate MCE specific activities behaved as a single gene and to map this gene with respect to E3 activity. The F_1 males were individually assayed for MCE and E3 activities and the F_1 females were backcrossed to RM2-6 males. The G_2 were assayed for both MCE and E3 activities.

2.2.5.4 *E9 Activity and Sh*

The presence versus absence of E9 activity was mapped to chromosome 4 by crossing virgin RM8 females with *Sh*/+ males. F_1 males expressing the *Sh* marker were crossed with virgin Q4 females (there is little or no recombination in *L. cuprina* males; (Foster *et al.*, 1980a; Foster *et al.*, 1991) and the backcross progeny were scored for *Sh* and E9 activity. Both the *Sh* marker strain and Q4 are non-staining for E9 on native PAGE (Table 2.1).

2.2.5.5 *E9 and E3 Activities*

E9 activity was mapped with respect to E3 activity using a cross between virgin Q4 females and LS2 males. F_1 males were assayed for E3 and E9 activities and F_1 females were backcrossed to Q4 males. The backcross progeny were scored for both esterase activities using α- and β-naphthylacetate as substrates after separation via native PAGE (Figure 2.1).

2.2.5.6 *E3 and E4 Activities and sv bu ra*

The E4 allozymic difference was mapped with respect to visible markers on chromosome 4 as well as E3 activity by crossing virgin Dua-91 females with *sv bu ra* males. F_1 females were backcrossed to *sv bu ra* males and the backcross progeny scored for the visible markers. Ten backcross progeny males from each phenotypic group were then individually crossed to virgin Dua-91 females. E3 and E4 activities were scored in three G_3 progeny of each sex for each cross.

2.3 Results

2.3.1 MCE Activity

The MCE specific activities of the seven strains surveyed varied from 1.6- to 33-fold less than the high RM8 line (Table 2.1), and were divided into three distinct groups, with no overlap in the activities between the groups. Thus the four low strains ranged from six to 15 pmol malathion/min/mg, the two intermediate strains gave activities of 136 to 140 pmol malathion/min/mg and the high RM8 line had a mean activity of 219 pmol malathion/min/mg. The activities of the low strains were all above the boiled enzyme control.

The MCE specific activity of RM8 was only half the value obtained for this line by Whyard *et al.* (1994b). The difference was not due to polymorphism in the RM8 line; numerous RM8 individuals were assayed and all gave specific activities within the same range (Table 2.1). The difference between the two studies could result from the use of different batches of ^{14}C-malathion. Amounts of monoacid impurities will vary between batches and these are known to potentiate malathion, presumably by inhibiting MCE activity (Umetsu *et al.*, 1977).

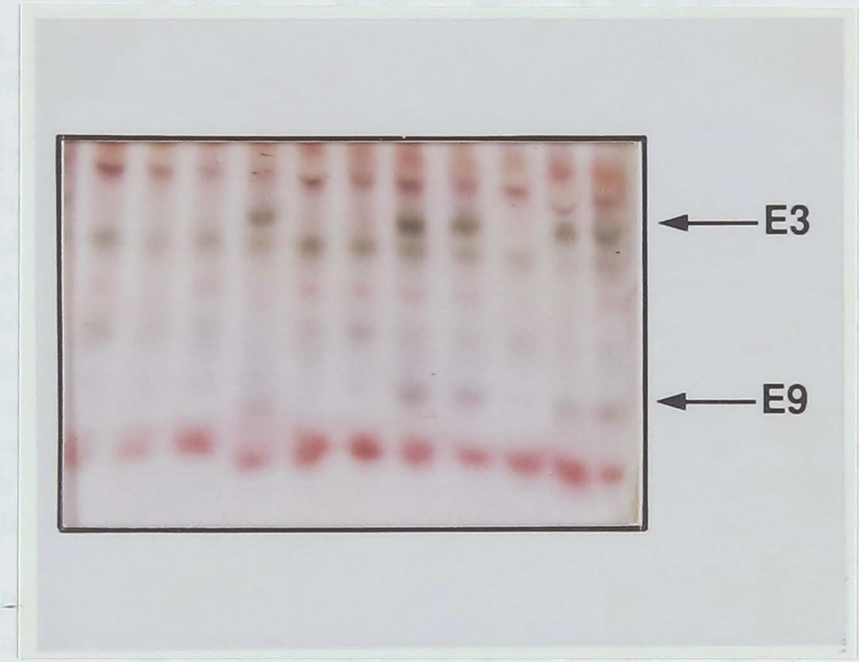


Figure 2.1 Native PAGE stained with α- and β–naphthylacetate showing the segregation of E3 and E9 in G_2 progeny from the mapping cross between Q4 and LS2.

Table 2.1 Properties of the strains used for mapping the esterase genes.

Strain	MCE Specific Activity		LD_{50}	Resistance Status	Esterase Phenotype[d] E3	E4	E9
Rm8	219.0 ± 5.0[a]	(High)	Female 345 (320 - 382)[b]	R[c]	+	S	+
			Male 261 (230 - 292)				
LS2	139.1 ± 3.5	(Intermediate)	Female 0.49 (0.44 - 0.54)	S	+	F	+
			Male 0.30 (0.26 - 0.33)				
sv bu ra	136.3 ± 5.5	(Intermediate)	< 2	S	+	S	+
Rm2-6	6.6 ± 0.2	(Low)	Female 0.39 (0.34 - 0.47)	S	-	F	-
			Male 0.24 (0.18 - 0.27)				
Q4	9.6 ± 0.8	(Low)	< 2	S	-	F	-
Sh	11.2 ± 1.4	(Low)	< 2	S	-	F/S	-
Dua-91	15.0 ± 0.6	(Low)	< 2	S	-	F	-

[a] pmol malathion/min/mg ± standard error.

[b] Dose (µg/µl) of malathion that kills 50% of individuals with 95% confidence intervals in parentheses.

[c] R=resistant, S=susceptible to malathion.

[d] +/- indicates staining or non-staining with α or β-naphthylacetate and F/S indicates fast or slow allozyme variants on native PAGE.

2.3.2 Malathion Resistance

The probit mortality curves for RM8, LS2 and RM2-6 were parallel (Figure 2.2). Surprisingly, the LS2 and RM2-6 lines have similar levels of resistance to malathion, although the difference in their MCE activities is 21-fold. The RM8 LD_{50} is almost 1000 times that of either the LS2 or RM2-6 lines, yet the difference in MCE activity between RM8 and LS2 is only 1.6-fold (Table 2.1). All the low and intermediate activity lines were completely susceptible to the discriminating dose of malathion. Females were slightly more resistant than the males in all three strains when treated with equal amounts of malathion, probably because females are generally larger than males.

2.3.3 Genetic Mapping

2.3.3.1 *High/Low MCE and E3 Activities*

High versus low MCE activity segregates as an allelic difference with little dominance. The F_1 progeny were all roughly midparental for MCE activity (78.6 to 113.6 pmol malathion/min/mg) and 325 of the 692 backcross progeny expressed this midparental phenotype, while the other 367 gave low MCE activities (Figure 2.3a). This is not significantly different from the 1:1 ratio of low to heterozygote activities that is expected if low and high MCE activities are controlled by alleles of a single locus (χ^2=2.54, $P \geq 0.05$).

The backcross progeny were also scored for E3 phenotypes and five recombinant individuals were recovered. All showed the MCE high/E3 non-staining phenotype. The map distance between *Rmal* (MCE activity) and *Rop1* (E3 activity) was therefore estimated as 0.7±0.3 map units.

2.3.3.2 *High/Intermediate MCE Activities and sv bu ra*

F_1 progeny from crosses between the high MCE activity RM8 line and the intermediate activity *sv bu ra* strain had MCE activities (143.4±2.6 pmol malathion/min/mg) lower than the midparental values and very close to those of the intermediate parents (136.3±5.5 pmol malathion/min/mg) (Figure 2.3b). Intermediate MCE activity thus appears dominant over high MCE activity. Intermediate homozygotes and intermediate/high heterozygotes could not be distinguished in the G_2 progeny because of this dominance (Figure 2.3b). The basis for the dominance of intermediate MCE activity over high MCE activity is not known, but may reflect the contribution of other genes to overall MCE activity (see Chapter 5 for further discussion). The high/intermediate MCE activities therefore could not be mapped with respect to the visible markers. Nor was it possible to overcome this problem by using other stains with multiple fourth chromosome markers; all five other such strains available also exhibited intermediate MCE activity (results not shown).

2.3.3.3 *Intermediate/Low MCE and E3 Activities*

The F_1 progeny from the cross between LS2 and RM2-6 expressed specific activities that lay between the two parental values but were clearly less than midparental (44.7±1.69 compared to 139.1±3.5 and 6.6±0.2 pmol malathion/min/mg for LS2 and RM2-6, respectively). Thus low MCE activity is partially dominant over intermediate MCE activity. One hundred and four backcross progeny had activities similar to those of the F_1 progeny and 97 showed low MCE activity (Figure 2.3c). The 1:1 ratio of backcross progeny shows that the low and intermediate MCE activities behave as alleles of a single gene ($\chi^2 = 0.24$, $P \geq 0.05$).

All 201 backcross progeny were also scored for E3 activity and no recombinants were recovered from the cross. Solving $(1-r)^{201} \geq 0.95$ and ≥ 0.99 (Mather, 1963)

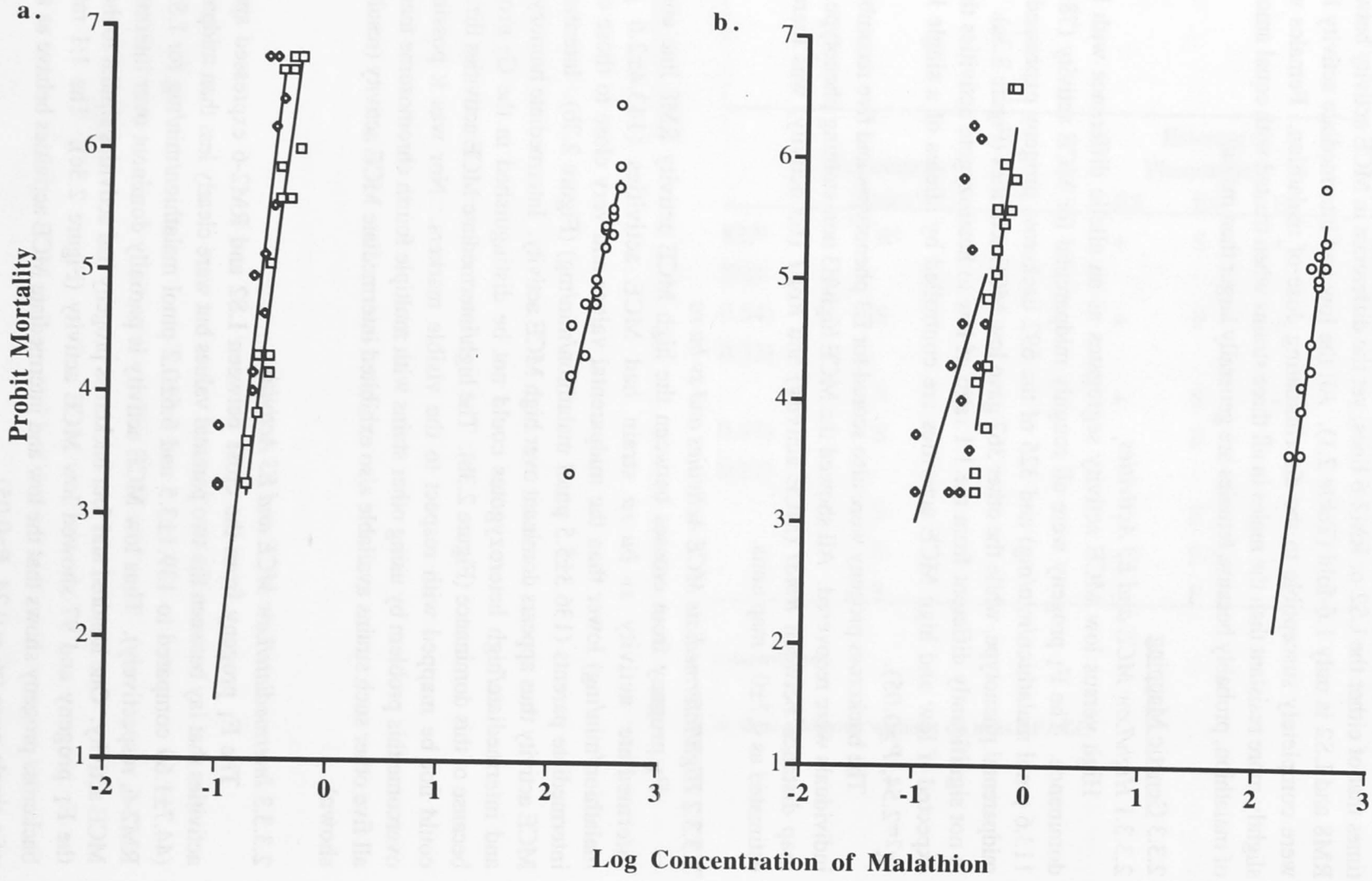

a.
b.
Probit Mortality
7
6
5
4
3
2
1
-2
-1
0
1
2
3
Log Concentration of Malathion

Figure 2.2 Mortality responses (probit scale) of the RM2-6 (low, ◊), LS2 (intermediate, □) and RM8 (high, o) lines to increasing concentrations of malathion for:

a. males and

b. females

Individual probit values (three experiments for each line) are given and the lines represent the predicted probit values.

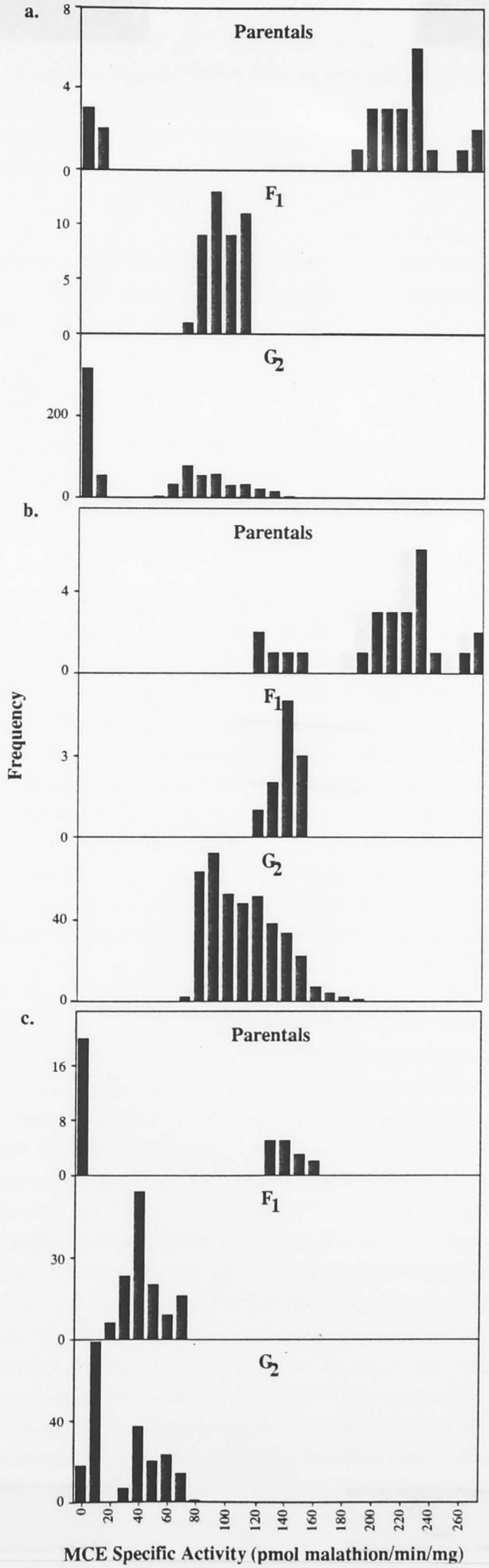
Figure 2.3 Distributions of MCE specific activity in individuals of the parental strains, F_1 and backcross progeny from three of the genetic crosses:

a. Q4 x RM8 (low verus high MCE activity),

b. *sv bu ra* x RM8 (intermediate versus high MCE activity),

c. LS2 x RM2-6 (intermediate versus low MCE activity).

For each, the first box represents the MCE specific activity of each parent, the second box those of the F_1 progeny and the third box those of the G_2 backcross progeny.


a.
Parentals
F1
G2
b.
Parentals
F1
G2
Frequency
c.
Parentals
F1
G2
0 20 40 60 80 100 120 140 160 180 200 220 240 260
MCE Specific Activity (pmol malathion/min/mg)

yields estimates of 0.026 map units and 0.005 map units for the upper bounds on the 95 and 99% confidence limits, respectively, of the estimate of the distance between the gene encoding the difference in MCE activities between low and intermediate and *Rop1*. On this basis the intermediate MCE activity is more likely to be allelic to *Rop1* than *Rmal*, although the probability of observing a recombinant between intermediate MCE activity and E3 non-staining activity based on the map distance of 0.7 map units between *Rop1* and *Rmal* is only 75 percent in this sample size.

2.3.3.4 *E9 Activity and Sh*

One hundred backcross individuals from the cross between RM8 and *Sh* were scored for the presence or absence of the *Sh* marker and E9 activity. In each case, E9 always segregated with the wild-type allele of the *Sh* locus (Table 2.2), placing E9 on chromosome 4.

2.3.3.5 *E9 and E3 Activities*

One recombinant was recovered from the 333 backcross individuals assayed for both E9 and E3 activities. The recombinant phenotype was E3 staining/E9 non-staining. E9 was thus mapped 0.3±0.3 map units from E3 (Table 2.2).

2.3.3.6 *E3 and E4 Activities and sv bu ra*

Foster *et al.* (1981) previously reported that the *bubbled wing* phenotype was not completely penetrant, and the lack of penetration was also observed in this experiment. Therefore, the esterase map distances were calculated using only the G_3 progeny generated from G_2 flies that expressed *bu*. A total of 162 G_3 progeny were assayed for E3 activity and 161 of these could also be scored for E4 activity. This enabled E3 and E4 genotypes to be assigned to 27 G_2 males, ten of which were + *bu ra*, six + *bu* +, seven *sv bu* +, and four *sv bu ra*. E3 and E4 were therefore mapped 3.7±1.5 map units and 4.3±1.6 map units from *bu*, respectively, and 6.1±1.9 map units from each other, on either side of *bu* (Table 2.2). The recombinational frequencies for *bu* and *sv*, and *bu* and *ra* estimated from this experiment are 16±1 and 20±1 percent, respectively, which are consistent with the map distances of Weller and Foster (1993) (24 and 20 map units, respectively, standard errors not available) albeit that these workers used different strains.

2.4 Discussion

The measurements of MCE specific activities from several malathion susceptible and resistant strains of *L. cuprina* reveal three distinct MCE phenotypes: low, intermediate and high activity. Both low and intermediate activities are associated with susceptibility to malathion although they differ 21-fold in MCE activities, from 6.6±0.2 pmol malathion/min/mg for low to 139.1±3.5 pmol malathion/min/mg for intermediate. The high MCE activity line is 1000-fold more resistant to malathion than either the intermediate or low MCE activity strains, yet it has only 1.6-fold greater MCE activity than the intermediate MCE strains. Thus, it appears that malathion resistance in *L. cuprina* is not just a simple linear function of MCE activity.

There are no other reports of any species showing polymorphism for MCE activities among malathion susceptible individuals, but susceptible strains of different species show a wide range of MCE activity. Susceptible strains of *Plodia interpunctella* share the same level of MCE specific activity as the low *L. cuprina* strains, about seven pmol malathion/min/mg (Beeman and Schmidt, 1982) compared with six to 15 for the low *L. cuprina* strains. On the other hand, susceptible strains of *Laodelphax striatellus* have approximately 50 times less specific activity and susceptible *C. tarsalis* have 100

times less MCE activity than low *L. cuprina* (Sakata and Miyata, 1994; Whyard *et al.*, 1994a), whereas susceptible *M. domestica* have about 80 times more MCE activity than intermediate *L. cuprina* (Shono, 1983).

Malathion resistance is also associated with elevated levels of MCE activity in the four species mentioned above, albeit, as with *L. cuprina*, the increases in resistance and activity are not proportional. The situation with *P. interpunctella* is similar to that of *L. cuprina*. Not only is the activity of susceptible individuals comparable to those of the low strains, but resistant individuals also show comparable increases in MCE activity (231±2.2 pmol malathion/min/mg compared with 219±5 for RM8; Beeman and Schmidt, 1982). However, the *L. cuprina* high line is ten times more resistant (94-fold for resistant versus susceptible *P. interpunctella* and 1000-fold for high versus low *L. cuprina*; Beeman and Schmidt, 1982). The relationship between MCE activity and malathion resistance in some strains of *L. striatellus* is similar to that of *P. interpunctella* because the increase in MCE activity (500-fold) is greater than the increase in malathion resistance (41-fold; Sakata and Miyata, 1994). On the other hand, resistant *C. tarsalis* are 150 times more resistant with only about 19-fold more MCE activity than susceptible mosquitoes (Whyard *et al.*, 1994a) and similarly, the Wakamatsu-m *M. domestica* strain is at least 1,600 times more resistant to malathion than the susceptible strain but only expresses a 5.3-fold more MCE activity (Shono, 1983). However, elevated levels of glutathione S-tranferase activity and AChE insensitivity also contribute to the extremely high level of malathion resistance in the Wakamatsu-m housefly strain.

These non-linear relationships between the increases in MCE activity and malathion resistance might be explained by either differences in MCE kinetic parameters other than specific activity (for example, the binding affinity for malathion (K_M)), or interactions with other genes (for example, the suppressor gene invoked by Plapp (1984) to explain aspects of OP resistance in *M. domestica*). There is no direct evidence to distinguish between these alternatives but it is relevant to note that there is no evidence to date for a non-MCE based mechanism of malathion resistance in *L. cuprina*. Raftos (1986) found no difference between RM and LS (the parental strains from which RM8, RM2-6 and LS2 were derived) in the sensitivity of AChE to malaoxon (the activated oxygen analogue of malathion), nor any difference in the rates of malathion penetration, excretion or storage. The production of malathion metabolites was not increased by the addition of NADPH or GSH, or inhibited by PBO, suggesting that mfos and GST activity are not involved in malathion resistance (Raftos, 1986). In contrast, MCE activity and malathion resistance were completely suppressed in RM with TPP (Hughes *et al.*, 1984; Raftos, 1986). Furthermore, Raftos (1986) also found resistance to malathion was tightly linked with high malathion carboxylesterase activity. Therefore, the only major mechanism of malathion resistance in *L. cuprina* appears to be increased MCE activity encoded by a single gene, *Rmal*.

On the basis of the data presented here, the map of chromosome 4 includes a cluster of at least three and possibly four esterase genes, depending on the allelism of intermediate MCE activity (Figure 2.4). The cluster includes *Rop1*, *Rmal* and *E9* and it lies approximately four map units proximal of the *bu* marker. Intermediate MCE also lies in the cluster and may be allelic to any one of the other three genes. The mapping data suggest that intermediate MCE activity could be an allele of *Rop1* since the E3 staining phenotype is always associated with intermediate MCE activity in the strains and crosses analysed here. However, it may also be an allele of *Rmal*, which encodes the low and high phenotypes. For further discussion refer to chapter 5.

Table 2.2 Summary of the genetic mapping results.

Mapping Experiment		Total Number of G_2 Progeny Scored		Number of Recombinants	Map Distance (map units ± S.E.)
1.	High/Low MCE and E3 activities	694		5	0.7 ± 0.3
3.	Intermediate/Low MCE and E3 activities	201		0	
4.	E9 activity and *Sh*	100		0	
5.	E9 and E3 activities	333		1	0.3 ± 0.3
6.	E3 and E4 activities and *sv bu ra*				
	E3 activity and *bu*	162		6	3.7 ± 1.5
	E4 activity and *bu*	161		7	4.3 ± 1.6
	E3 and E4 activities	161		10	6.1 ± 1.9
	sv-bu-ra	1535	*sv-bu*	238	15.5 ± 0.9
			bu-ra	310	20.2 ± 1.0
			sv-ra	422	27.5 ± 1.1

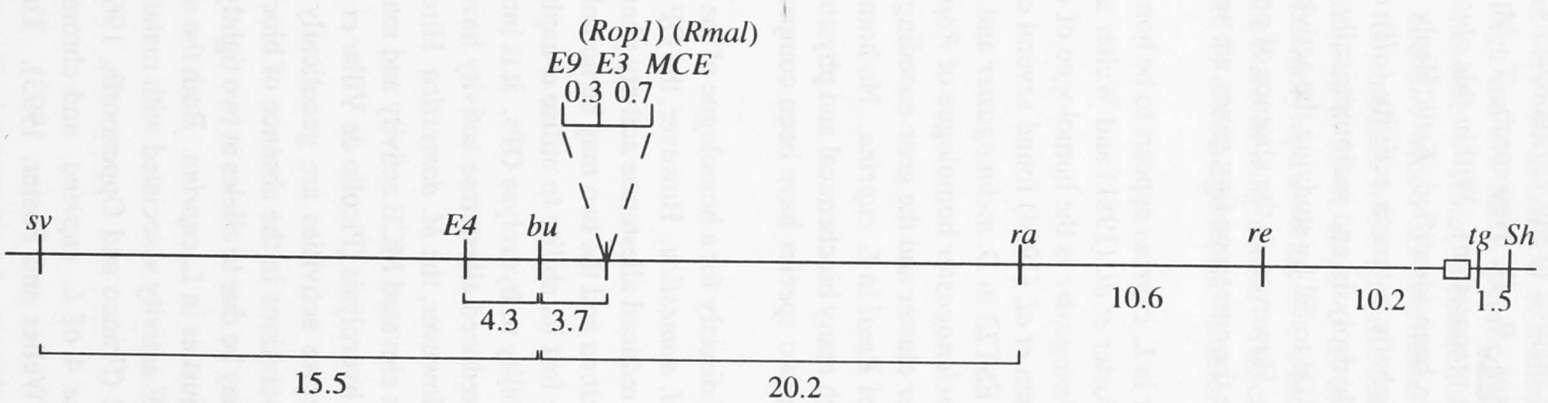


Figure 2.4 Revised linkage map of the left arm of chromosome 4 of *L. cuprina* showing the positions of *Rop-1* (E3), *Rmal* (MCE), *E9*, *E4* and the visible markers used in the mapping experiments. Numerals indicate percent recombination between the nearest markers. The position of the centromere with respect to the visible markers, as determined by Foster *et al.* (1980b), is indicated by □.

Two clusters of esterase genes are known in *D. melanogaster*. These clusters are designated the α and β clusters according to the preference of most of their members for α- or β-naphthylacetate as *in vitro* substrates. The β-esterase genes, *Est6* and *EstP* are tightly linked at subdivision 69A1-5 on chromosome 3L (Collet *et al.*, 1990). The α-esterase cluster has been localised to subdivision 84D3 to E2 on chromosome 3R (Spackman *et al.*, 1994) and molecular analysis of this region reveals up to 11 esterase genes (R.J.Russell, C. Robin, P. Kostakos, R. D. Newcomb, T. M. Boyce, K. M. Medveczky, D. L. Hartl, and J.G.Oakeshott, in preparation). Within this cluster, two separate genes encoding esterase isozymes have been identified, *Est9* (Healy *et al.*, 1991) and *Est23* (Spackman *et al.*, 1994). Aliphatic esterase activity (*ali*) determined by a manometric assay for methylbutyrate hydrolysis and radiometrically determined *Mce* activities have also been localised to 84D3 to E2 by studying the activities of deficiency heterozygotes (Spackman *et al.*, 1994). However, in the absence of polymorphisms for both *ali* and *Mce* it is not clear whether the corresponding genes are separate from each other or from *Est9* and *Est23*.

The MCE/E3/E9 esterase cluster in *L. cuprina* appears to be homologous to the α-esterase cluster of *D. melanogaster*. Foster *et al.* (1981) and Weller and Foster (1993) showed that chromosome 3R in *D. melanogaster* is the homologue of chromosome 4 in *L. cuprina*. More specifically, Spackman *et al.* (1994) found several close biochemical and physiological similarities between EST23 in *D. melanogaster* and E3 in *L. cuprina*, and postulated that *Est23* was the *D. melanogaster* homologue of *Rop1*. MCE activity was also mapped to the *D. melanogaster* cluster and the gene encoding *D. melanogaster* MCE activity may be the homologue of *Rmal* in *L. cuprina*. No homologue of E9 has been found in *D. melanogaster*, although many biochemical and physiological properties of the esterases so far identified in the two species have been compared (Healy *et al.*, 1991; Parker *et al.*, 1991).

L. cuprina has not been assayed directly for a homologue of the aliphatic esterase characterised in *D. melanogaster* and *M. domestica*. However, the OP resistant allele of *Rop1* shows some similarities with the reduced aliesterase activity phenotype associated with an OP resistance gene in *M. domestica* and the two may be homologous. The non-staining E3 phenotype appears to have lost its ability to utilise α-naphthylacetate as an artificial substrate, while gaining the ability to hydrolyse OPs. It is intriguing that some of the *M. domestica* strains that show reduced aliesterase activity have enhanced MCE activity and resistance to malathion. However, the *M. domestica* Hirokawa strain is an informative exception because it shows elevated MCE activity and malathion resistance but normal levels of α-naphthylacetate hydrolysis (Picollo de Villar *et al.*, 1983). These results suggest that MCE and aliesterase activities are genetically separable in *M. domestica* and concur with earlier conclusions in the absence of biochemical data that resistance to parathion and malathion may be due to alleles at two tightly linked loci in *M. domestica* (Nguy and Busvine, 1960), just as in *L. cuprina*. Both the aliesterase activity associated with OP resistance and MCE activity associated with malathion resistance in *M. domestica* map to chromosome 2 (Franco and Oppenoorth, 1962; Shono, 1983), which is homologous to chromosome 4 of *L. cuprina* and chromosome 3R of *D. melanogaster* (Foster *et al.*, 1981; Weller and Foster, 1993). These data and the precedents of esterase clusters on homologous chromosomes in *D. melanogaster* and *L. cuprina* suggest that MCE, aliesterase and some other esterase genes may well be clustered in *M. domestica*.

2.5 Summary

Three distinct malathion carboxylesterase (MCE) phenotypes have been identified among seven strains of *L. cuprina*. The high MCE activity phenotype shows 1.6- and 33-fold more MCE specific activity than the intermediate and low MCE activity phenotypes, respectively. Flies with high MCE activity are 1000-fold more resistant to malathion than flies with either low or intermediate MCE phenotypes, which are equally susceptible. High and low MCE specific activities are allelic and encoded by the *Rmal* gene on chromosome 4. *Rmal* is located within one map unit of two other esterase genes, *Rop1* and *E9*, which are implicated in resistance to other organophosphate insecticides. Intermediate MCE specific activity is also co-inherited with this cluster, although its allelism to *Rmal*, *Rop1* or *E9* is unclear. The cluster does not contain the gene for the haemolymph esterase E4, which maps 6.1 map units from *Rop1*, on the other side of the *bubbled wing* marker. The cluster appears to be homologous to part of a tandem array of 11 esterase genes on chromosome 3R of *D. melanogaster*.

CHAPTER 3

BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISATION OF MCE ACTIVITIES IN MALATHION SUSCEPTIBLE AND RESISTANT LINES

3.1 Introduction

Detoxication of most organophosphorus insecticides (OPs) by esterases involves binding, and in some cases cleavage of the phosphoester linkages. Resistance in both *Culex quinquefasciatus* and *Myzus persicae* depends upon gene amplification that increases the production of esterases that bind and essentially sequester the OP (Devonshire and Moores, 1982; Section 1.3.2.3.2). Resistance in *C. quinquefasciatus* is due to an abundance of esterase B1, which binds to OPs without detectable cleavage (Mouchès *et al.*, 1986). Although the E4 isozyme from *M. persicae* hydrolyses OPs very slowly (turnover rate of 0.1 min^{-1} for malaoxon; Devonshire and Moores, 1982; Field *et al.*, 1988) it comprises 3% of total soluble protein. The slow cleavage of the OP by E4 implies phosphatase activity, however the enzyme still behaves as a typical carboxylesterase *in vitro*; it hydrolyses carboxyl esters, is inhibited by low concentrations of paraoxon and is uninhibited by eserine sulphate (Devonshire and Moores, 1982). By contrast, some other cases of resistance to the same OPs have been attributed to mutations that alter the structure of a particular esterase so that it acquires efficient phosphatase activity, but in the process loses the ability to hydrolyse carboxylesters (Section 1.3.2.3.3). The best studied cases attributed to this mechanism are the aliesterase of *Musca domestica* (Oppenoorth and van Asperen, 1960) and the E3 isozyme in *Lucilia cuprina* (Hughes and Raftos, 1985) although direct kinetic data to demonstrate phosphatase activity are not yet available for either species.

A small number of OPs like malathion contain one or more carboxylester bonds in addition to the phosphoester linkage (Table 1.1). Malathion can be hydrolysed at either or both of its two carboxylester bonds into the less toxic malathion mono- and diacids (Eto, 1974). Most examples of esterase mediated malathion resistance involve increased MCE activity. In most cases it is not known whether the increase in MCE activity is due to changes in the structure or amount of enzyme (Section 1.4.5). However, in *Culex tarsalis* resistance is known to be associated with a structurally unique MCE not found in the susceptible strain (Whyard *et al.*, in press). The MCE from the resistant strain is not abundant (0.02% of total soluble protein) but it has a high turnover rate (turnover rate of 17 min^{-1} for malathion; Whyard *et al.*, in press).

Malathion resistance in *L. cuprina* is also conferred by increased MCE activity (Hughes *et al.*, 1984; Raftos and Hughes, 1986; Whyard *et al.*, 1994b; Section 2.3.2). Recently, an MCE enzyme from the resistant RM strain has been purified and characterised (Whyard *et al.*, 1994b; Whyard and Walker, 1994; Section 1.5.2). The enzyme shows similarities with the novel MCE found in resistant *C. tarsalis* as it is not abundant (0.05% of total soluble protein) and is a highly efficient catalyst (turnover rate of 46 min^{-1} for malathion; Whyard *et al.*, 1994b). Another resistant strain, der-L, was shown to have about ten-fold less of the same enzyme and its LD_{50} for malathion was also about ten-fold lower than RM (Whyard and Walker, 1994). However, the differences between the MCEs of resistant and susceptible strains have not been investigated until now.

Chapter 2 reported three distinct MCE activity phenotypes in crude homogenates of adults from one resistant and six susceptible strains of *L. cuprina*. These phenotypes are denoted low, intermediate, and high. This chapter describes the physiological and biochemical characterisation of MCE activities from a representative iso-chromosomal line for each of the three MCE activity phenotypes. The results suggest that the predominant MCE activity present in the malathion resistant high MCE activity line is structurally different from those in the malathion susceptible intermediate and low MCE activity lines.

3.2 Materials and Methods

3.2.1 Strains

The derivation of the three iso-chromosome 4 lines was described in Whyard *et al.* (1994b) and Section 2.2.1. The low, malathion susceptible (RM2-6) and high, malathion resistant (RM8) lines were derived from the RM line. The intermediate line (LS2) was derived from a field strain (LS) and is susceptible to malathion (Section 2.3.2; Table 3.1). The low, intermediate and high MCE activity lines are hereafter referred to as the low, intermediate and high lines, respectively.

Larvae were raised on a combination of sheep liver and meat meal media at 27°C (Foster *et al.*, 1981) and adults were maintained on a diet of sugar and water prior to assaying.

3.2.2 MCE Assay

MCE activity was assayed using the radiometric partition method of Ziegler *et al.* (1987) as modified by Whyard *et al.* (1994b) (Section 2.2.2). MCE homogenisation buffer contains 10mM imidazole HCl, pH7.0, 1% Triton X-100 (v/v), 1mM PTU and 1mM DTT. Protein concentrations were determined using the method of Bradford (1976) with ovalbumin as the standard (Section 2.2.2).

3.2.3 Developmental Profile

MCE specific activities were measured at each life stage and at several different times within each life stage for the low and high lines. Each sample assayed contained one hundred eggs, fifteen first instar larvae, five second instar larvae or one individual from older larvae, pupae or adults. Samples of day four larvae and 1 - 3 day old adults from the intermediate line were also assayed. All samples were homogenised in 1ml MCE homogenisation buffer and immediately assayed for MCE activity. At least three replicate samples from different population cages were assayed for each time point from each line, and all samples were assayed in duplicate. There was no difference in MCE specific activity between adult males and females of each line (data not shown), and these data have been combined for the developmental profiles.

3.2.4 Tissue Distributions

MCE activities were also determined for several individual tissues from day four larvae (wandering third instar) and 1 - 3 day old adults for the low and high lines. Crops were dissected from three and five day old larvae. All tissues except the haemolymph were dissected into Ringer's solution (0°C; Roberts, 1986) containing 1mM DTT, homogenised in 500µl MCE homogenisation buffer and assayed immediately for MCE activity. Haemolymph was collected with a fine glass needle (about 3µl per fly for both

larvae and adults, representing about 50% of the total volume of haemolymph (5.6±1.3µl) in a fly) and made up to a final volume of 500µl with MCE homogenisation buffer. For the larval bodywall and adult head, thorax and abdomen each sample comprised the material from five individuals. Each sample of the other tissues (refer to Table 3.2) comprised the material from 10 individuals. Three replicate samples from different population cages were assayed in duplicate for each tissue from each line. The liver and meat meal media were also tested for MCE specific activity, to rule out the possibility that enzymes in the food stored in the crop contributed to crop MCE activity. Both the liver and meat meal media contained insignificant amounts of MCE specific activity (results not shown).

3.2.5 Inhibitor Studies

Five esterase inhibitors were tested for their effects on MCE activity in homogenates of day four larvae and 1 - 3 day old adults for each of the three lines. Individual flies were homogenised in 600µl MCE homogenisation buffer and aliquots of high (15µl), intermediate (15µl) and low (75µl) line homogenates were pre-incubated with an inhibitor (or the inhibitor solvent, ethanol, for the controls) for 30 minutes at 25°C before assaying for MCE activity. The inhibitors used were the OPs paraoxon ($2x10^{-5}$, $2x10^{-6}$ and $2x10^{-7}$M) and diisopropylfluorophosphate (DFP; 10^{-4} and 10^{-5}M), the carboxylesterase inhibitor triphenylphosphate (TPP; $3x10^{-5}$, $3x10^{-6}$ and $3x10^{-7}$M), the cholinesterase inhibitor eserine sulphate (10^{-4}M), and 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP; $2x10^{-6}$M), which is a potent inhibitor of some carboxylesterases (Hammock *et al.*, 1984; Campbell *et al.*, 1992). Three replicates from different population cages were assayed in duplicate for each inhibitor and life stage for the three lines.

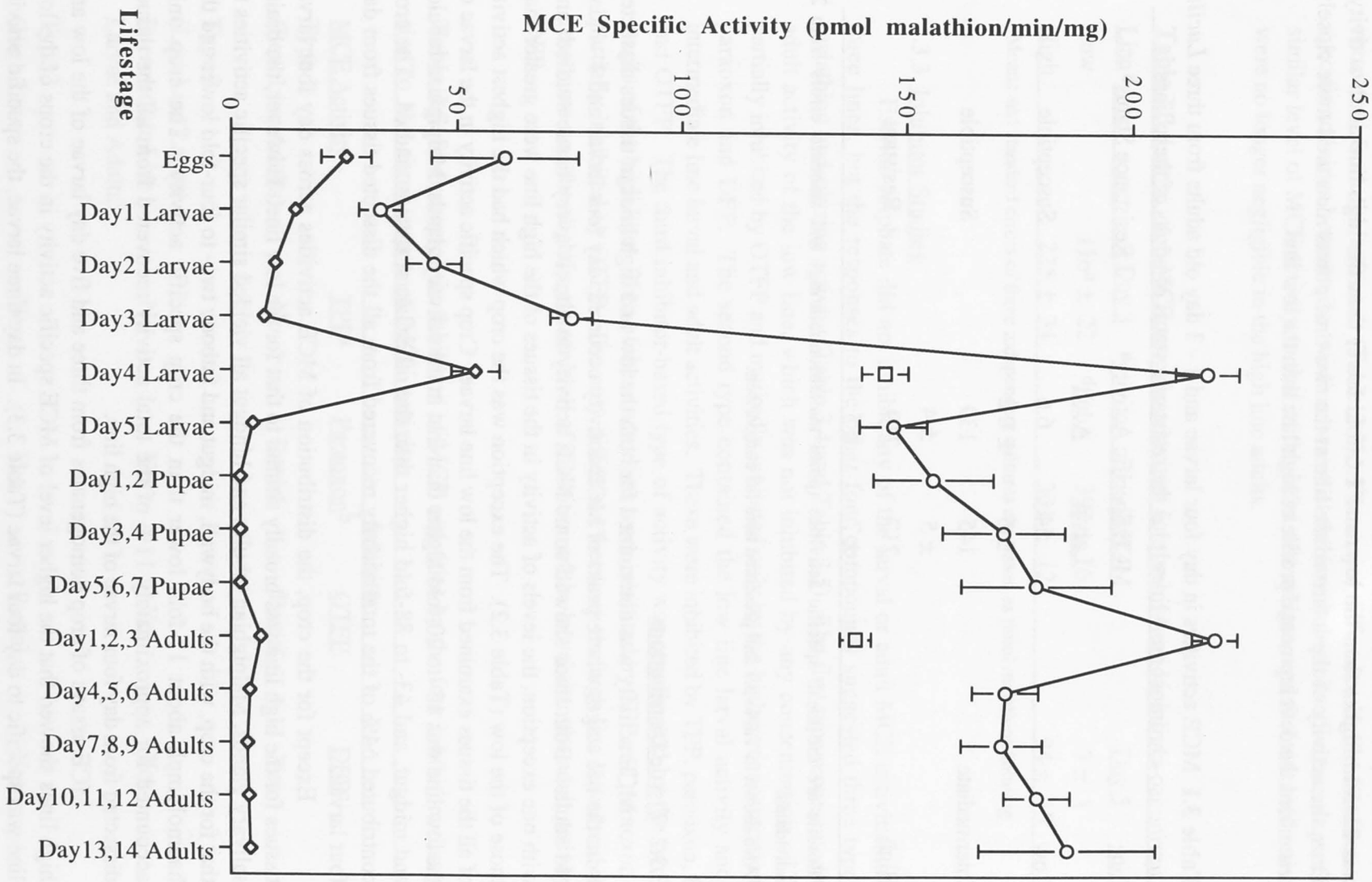
3.3 Results

3.3.1 Developmental Profile

The developmental profile of MCE specific activity differed between the low and high lines (Figure 3.1). In the low line MCE specific activity was higher in eggs than the activity in one, two or three day old larvae, but activity then peaked in day four larvae (54.0±5.3 pmol malathion/min/mg; Table 3.1). MCE specific activity fell markedly to negligible levels in pupae (2.1±0.3 pmol malathion/min/mg for mid pupae) and rose slightly in adults (6.6±0.2 pmol malathion/min/mg), albeit remaining lower than the MCE specific activity in eggs or larvae. In the high line MCE specific activity was also higher in eggs than early larvae, and a peak of MCE specific activity also occurred in day four larvae (216.8±7.0 pmol malathion/min/mg). This peak of activity was four-fold higher than that of the low line. Unlike the low line however, MCE specific activity in the high line fell ~~slightly~~ during pupal life (172±13.5 pmol malathion/min/mg for mid pupae), so it was 80 times higher than that found in the low line at ~~this~~ stage. Activity in the high line then rose again in 1 - 3 day old adults (219±5 pmol malathion/min/mg) to levels that were equivalent to the larval peak, which was 33-fold higher than the corresponding low line activity.

MCE specific activity was characterised from the intermediate line for the larval and adult peaks of activity observed in the low and high lines (Figure 3.1; Table 3.1). Activity in day four larvae of the intermediate line (145.4±5.2 pmol malathion/min/mg) was 2.7-fold higher than the low and 1.5-fold lower than the high line activity at this

Figure 3.1 Developmental profile of MCE specific activity (pmol malathion/min/mg) for the low (RM2-6, ◊), intermediate (LS2, □) andhigh (RM8, O) lines. Symbols represent the mean result for three independent cultures. Standard errors are shown only where they exceed the size of the symbol. Adult male and female data have been combined.


MCE Specific Activity (pmol malathion/min/mg)
0
50
100
150
200
250
Lifestage
Eggs
Day1 Larvae
Day2 Larvae
Day3 Larvae
Day4 Larvae
Day5 Larvae
Day1,2 Pupae
Day3,4 Pupae
Day5,6,7 Pupae
Day1,2,3 Adults
Day4,5,6 Adults
Day7,8,9 Adults
Day10,11,12 Adults
Day13,14 Adults

stage. The level of MCE specific activity in early adults of the intermediate line (139.1±3.5 pmol malathion/min/mg) was equivalent to the activity in day four larvae, and was 21-fold higher than the low, and 1.6-fold lower than the high line adult activity. Thus, the activity of the intermediate line at the two time points measured more closely resembled the developmental profile of high line than the low line.

Table 3.1 MCE activities in day four larvae and 1 - 3 day old adults from three *Lucilia cuprina* iso-chromosome 4 lines, plus the resistance status of adults of these lines.

Line	MCE Specific Activity[a]		Resistance Status[b]
	Larvae	Adult[b]	
Low	54 ± 5	6.6 ± 0.2	Susceptible
Intermediate	145 ± 5	139 ± 4	Susceptible
High	217 ± 7	219 ± 5	Resistant

[a]Results are the mean specific activities (pmol malathion/min/mg) and standard errors from 20 individuals.

[b]Also shown in Table 2.1 and presented here for comparison.

3.3.2 Tissue Distributions

MCE activity was determined for both the low and high lines in tissues dissected from the life stages where peaks of MCE activity occurred; day four larvae and 1 - 3 day old adults. Both lines showed some MCE activity in almost every tissue studied, and with one exception, the levels of activity in the tissues of the high line were greater than those of the low (Table 3.2). The exception was the crop which had the highest activity of all the tissues examined from the low line larvae. Crop specific activity in the larvae of the low line was 15- to 20-fold higher than that in the salivary glands, Malpighian tubules and midgut, and 43- to 88-fold higher than the other larval tissues studied. The crop contributed 64% of the total activity recovered from all the dissected tissues from day four larvae.

Except for the crop, the distribution of MCE activities across day four larval tissues for the high line was broadly similar to that for the low line. However, the brain, salivary glands, Malpighian tubules and hindgut all yielded similar specific activities to that for the crop, with the bodywall, midgut and fatbody two- to four-fold lower and the haemolymph about 17-fold lower than the crop specific activity. The crop only accounted for approximately 11% of the total activity recovered from all the tissues dissected from day four larvae of the high line.

MCE assays of crop homogenates from three and five day larvae of the low and high lines showed that the higher level of MCE specific activity in the crops of the low line was specific to day four larvae (Table 3.3). In day three larvae, the specific activity in the crop was two-fold lower in the low line than the high line and in day five larvae it was over 13-fold lower.

MCE activity was low in all tissues dissected from adults of the low line. The head and midgut yielded the highest specific activity values, while no activity was recovered from the antennae (Table 3.2). The major difference in the distribution of specific activities between the two lines was that the female reproductive tract expressed a similar level of MCE activity to that of the head and midgut, and the antennal activities were no longer negligible in the high line adults.

Table 3.3 Larval crop MCE specific activities for the low and high lines.

Line	Day 3	Day 4	Day 5
Low	116[a] ± 22	392 ± 16	7 ± 1
High	225 ± 34	301 ± 12	94 ± 15

[a]Means and standard errors of three independent cultures expressed as pmol malathion/min/mg

3.3.3 Inhibitor Studies

Eserine sulphate did not inhibit any of the larval or adult MCE activities of the three lines, but the responses to the other four compounds suggested three types of malathion hydrolysing activity (Table 3.4 and Table 3.5). The first type represented only adult activity of the low line, which was not inhibited by any concentration of TPP, partially inhibited by OTFP and markedly inhibited only by the highest concentrations of paraoxon and DFP. The second type contained the low line larval activity and the intermediate line larval and adult activities. These were inhibited by TPP, paraoxon, DFP and OTFP. The third inhibitor-based type of activity was represented by the two life stages of the high line. Both of these activities were inhibited by TPP and paraoxon and partially inhibited by OTFP. The only difference between the high line larval and adult responses was the slightly greater level of inhibition of the larval than the adult activity by DFP.

Table 3.5 Summary of the inhibition of MCE activities by the four inhibitors that give rise to the three types of MCE activity.

	Inhibitor			
MCE Activity	TPP[a]	Paraoxon[b]	OTFP	DFP[c]
Type 1 (Low Line Adults)	No	Partial	Partial	Yes
Type 2 (Low Line Larvae Intermediate Line Larvae and Adults)	No	Yes	Yes	Yes
Type 3 (High Line Larvae and Adults)	Yes	Yes	Partial	Yes

[a]3×10^{-6}M TPP, [b]2×10^{-7}M Paraoxon and [c]10^{-4}M DFP

Table 3.2 MCE activities and specific activities for various tissues from day four larvae and 1 - 3 day old adults for the low and high lines.

Tissue	Low Line		High Line	
	Activity[a]	Specific Activity[b]	Activity	Specific Activity
Larval				
Brain	0.1 ± 0.1	4 ± 1	0.9 ± 0.3	177 ± 17
Salivary Glands	0.2 ± 0.1	16 ± 3	4.6 ± 0.9	324 ± 4
Haemolymph	0.2 ± 0.0	4 ± 0	1.0 ± 0.4	18 ± 1
Malpighian Tubules	0.2 ± 0.0	20 ± 12	3.9 ± 1.1	473 ± 26
Crop	4.4 ± 0.9	392 ± 16	3.3 ± 0.3	301 ± 12
Midgut	0.9 ± 0.2	25 ± 3	4.5 ± 0.9	100 ± 9
Hindgut	0.1 ± 0.1	8 ± 1	3.4 ± 0.4	241 ± 10
Fatbody	0.4 ± 0.1	8 ± 1	4.5 ± 0.9	97 ± 2
Bodywall	0.4 ± 0.1	9 ± 1	5.1 ± 1.2	82 ± 6
Adult				
Head	0.3 ± 0.2	7 ± 2	7 ± 0.6	110 ± 12
Thorax	0.3 ± 0.1	2 ± 1	6 ± 0.9	21 ± 3
Abdomen	0.3 ± 0.2	3 ± 1	5 ± 0.7	59 ± 7
Antennae	0	0	0.4 ± 0.1	44 ± 5
Brain	0.1 ± 0	4 ± 2	1.0 ± 0.2	76 ± 1
Thoracic Muscle	<0.05	1 ± 0	0.7 ± 0.1	19 ± 1
Haemolymph	<0.05	2 ± 1	0.1 ± 0.0	19 ± 10
Malpighian Tubules	<0.05	2 ± 0	0.7 ± 0.2	56 ± 4
Midgut	0.1 ± 0	7 ± 1	2.2 ± 0.3	111 ± 8
Hindgut	<0.05	3 ± 1	1.0 ± 0.1	74 ± 10
Male Reproductive Tract	<0.05	2 ± 1	0.7 ± 0.3	48 ± 6
Female Reproductive Tract	<0.05	2 ± 0	2.2 ± 0.6	113 ± 7

[a]Means and standard errors of three independent cultures are expressed as pmol malathion/min/individual

[b]Specific activities are expressed as pmol malathion/min/mg

Table 3.4 The effect of inhibitors on MCE activities in crude homogenates of each of the three lines for day four larvae and 1 - 3 day old adults.

Line	TPP			Paraoxon			OTFP	Eserine	DFP	
	$3x10^{-5}M$	$3x10^{-6}M$	$3x10^{-7}M$	$2x10^{-5}M$	$2x10^{-6}M$	$2x10^{-7}M$	$2x10^{-6}M$	$10^{-4}M$	$10^{-4}M$	$10^{-5}M$
Larvae										
Low	10[a]	39	59	1.7	3.9	16	8	100	1.5	2.2
	± 0	± 13	± 9	± 0.5	± 0.5	± 2	± 3	± 0	± 0.5	± 0.3
Intermediate	15	57	89	0.8	1.5	2	3	98	0.5	1.2
	± 1	± 3	± 3	± 0.5	± 0.4	±1	± 1	± 0	± 0.2	± 0.5
High	3	4	9	2.0	3.8	19	48	88	0.6	3.3
	± 1	± 1	± 1	± 0.8	± 0.3	± 1	± 2	± 3	± 0.2	± 1.2
Adults										
Low	95	100	100	2.8	52	64	41	100	6.1	31
	± 5	± 0	± 0	± 2.2	± 15	± 17	± 0	± 0	± 2.6	± 3
Intermediate	19	77	100	0.2	0.8	1.2	3	100	0	0.2
	± 3	± 5	± 0	± 0.1	± 0.4	± 0.4	± 1	± 0	± 0	± 0.2
High	0	4	28	0.9	6.4	22	39	100	0	33
	± 0	± 2	± 10	± 0.6	± 1	± 5	± 9	± 0	± 0	± 10

[a]Results are the mean and standard errors of three homogenates from different cultures expressed as percent specific activity of uninhibited control samples.

3.4 Discussion

Substantial changes in MCE activity occur throughout the life cycle of *L. cuprina*. There are two equal peaks of activity in day four larvae and 1 - 3 day old adults of the high line. However, MCE activity in the low line declines drastically after the larval peak and only rises slightly in early adults. MCE activity was only examined in the intermediate line in day four larvae and 1 - 3 day old adults, but its activity at these times were roughly equal, and so the developmental profile of MCE in this line may resemble that of the high line. The distinct developmental profile in the low line suggests that MCE activity may be regulated differently in this line than the other two. The developmental profile for the high line resembles those found in both susceptible and resistant lines of *C. tarsalis*, where a peak of activity in third instar larvae is followed by a drop in activity in pupae, with a second peak of activity in newly emerged adults (Whyard *et al.*, 1994a).

Almost all of the tissues assayed in day four larvae and early adults of the low and high lines expressed some level of MCE activity. In general, tissues involved in digestion, excretion and neurological function from both lines displayed higher levels of MCE activity than other tissues assayed. However, there was large variation in activity across tissues. The three major strain differences were the relatively high levels of specific activity in the crop of the low line larvae (1.3-fold higher than the high line), and the female reproductive tract (57-fold higher than the low line) and antennae (44 pmol malathion/min/mg compared to undetectable levels in the low line) of the high line. The crop was the only tissue assayed where MCE activity was higher in the low line than in the high line. The higher level of crop activity in the low line appears to be specific to day four larvae as three and five day larvae showed higher crop activities in the high line. As the low line is susceptible to malathion, the larval crop activity of the low line apparently does not contribute to malathion resistance.

No other example of such a transient crop-specific expression in other organisms characterised for MCE activity has been reported. However, there is a precedent for some tissue specific differences in MCE activity between susceptible and resistant strains. In *C. tarsalis*, MCE activity in the midgut is almost 25 times greater in resistant than susceptible larvae, while the difference in most other tissues is only five- to six-fold (Whyard *et al.*, 1994a). Notwithstanding any structural differences that might exist between the MCEs of susceptible and resistant strains, it seems that tissue specific expression can also differ.

Three structurally distinct types of MCE activity can be inferred from the pattern of esterase inhibition across the life stages and lines of *L. cuprina* assayed. One type of activity occurs in the adults of the low line, another in the larvae of the low line plus both larvae and adults of the intermediate line, and a third in both larvae and adults of the high line. All three types may be classified as B-esterases because of their inhibition by OPs, and as carboxylesterases because of their insensitivity to eserine sulphate (Aldridge, 1993). On the criteria of Healy *et al.* (1991), the activity in the low line adults would be further classified as a subclass II carboxylesterase because it is only partially inhibited by paraoxon at $2x10^{-6}$M, while the other two types would both be subclass I carboxylesterases due to their sensitivity to concentrations of paraoxon as low as $2x10^{-7}$M ~~(Healy *et al.*, 1991)~~.

It is important to note that the MCE activity of the high line is more sensitive to inhibition by TPP than the first two types of MCE activity (Figure 3.2). Early studies also found malathion resistant strains to be more sensitive to TPP in *C. tarsalis* and *M. domestica* where malathion resistance is associated with elevated MCE activity (Plapp

and Eddy, 1961; Plapp *et al.*, 1963). In contrast, Raftos (1986) and Whyard *et al.* (1994b) had previously reported no differences in the TPP inhibition of MCE among two susceptible and one resistant strain of *L. cuprina* (LS, der-S and RM, respectively). However, they used a concentration of TPP ($3x10^{-5}M$) ten times that which is necessary to discriminate between MCE activity types in this species ($3x10^{-6}M$).

The presence of different inhibitor-based types of MCE activity in the larvae and adults of the low line suggests that at least two types of MCE enzymes differing in both structure and regulation can occur within one iso-chromosomal line. There is also evidence for more than one form of MCE within strains of *C. tarsalis* (Whyard *et al.*, in press) and *M. domestica* (Welling and Blaakmeer, 1971). In both of these species, two MCE activities were recovered from a malathion resistant strain, one specific to the resistant strain and the other common to both the susceptible and resistant strains. Whyard and Walker (1994) purified only one MCE enzyme from two resistant strains of *L. cuprina*, but some MCE activity was eluted with the void volume of their gel filtration column (S. Whyard, personal communication), and this may have represented a distinct type of MCE.

How can the differences in MCE activity levels between the high, intermediate and low lines be interpreted? The low and intermediate lines contain MCEs which differ with respect to their inhibitor responses in adults but not larvae. Therefore, a difference in the expression of the same structurally defined enzyme may account for the different activity levels in larvae of the two lines. A second change in expression may account for the seemingly structural difference between larvae and adults of the low line, effectively turning off expression of the larval enzyme in adults of the low line. The predominant MCE in the malathion resistant high line differs from both intermediate and low line MCEs in its inhibitor profile. Given the possibility of multiple MCE genes or enzymes within strains, the changes in MCE associated with malathion resistance could result from either a regulatory change increasing the amount of an MCE previously expressed at negligible levels or ~~p~~a structural mutation in an MCE gene producing a novel MCE protein.

3.5 Summary

Chapter 2 described three MCE phenotypes in *L. cuprina* adults: low, intermediate and high. The MCE specific activity of adults with the intermediate and high phenotypes are 21- and 33-fold higher, respectively, than that of the low phenotype. The results of this chapter show that MCE activity also peaks in day four larvae from three iso-chromosomal lines representative of these phenotypes. The MCE activity of the low line larvae is only 2.7- and 4-fold lower than those of the intermediate and high line larvae, respectively. The relatively high MCE activity of the low line larvae is largely explained by the crop activity. Assays in the presence of esterase inhibitors reveal three distinct types of MCE activity across the three lines and two developmental stages. Activity in the low line adults is not completely inhibited by paraoxon and is classified as a subclass II carboxylesterase. The MCE activities in the larvae of the low and adults and larvae of the intermediate line are inhibited by low concentrations of paraoxon, classifying them as subclass I carboxylesterases. The MCE activities in the high line larvae and adults are also classified as subclass I carboxylesterases, but they are more sensitive to inhibition by TPP than those in the other two lines. These data suggest that MCE in the malathion resistant high line may be structurally different from the MCEs in the malathion susceptible intermediate and low lines.

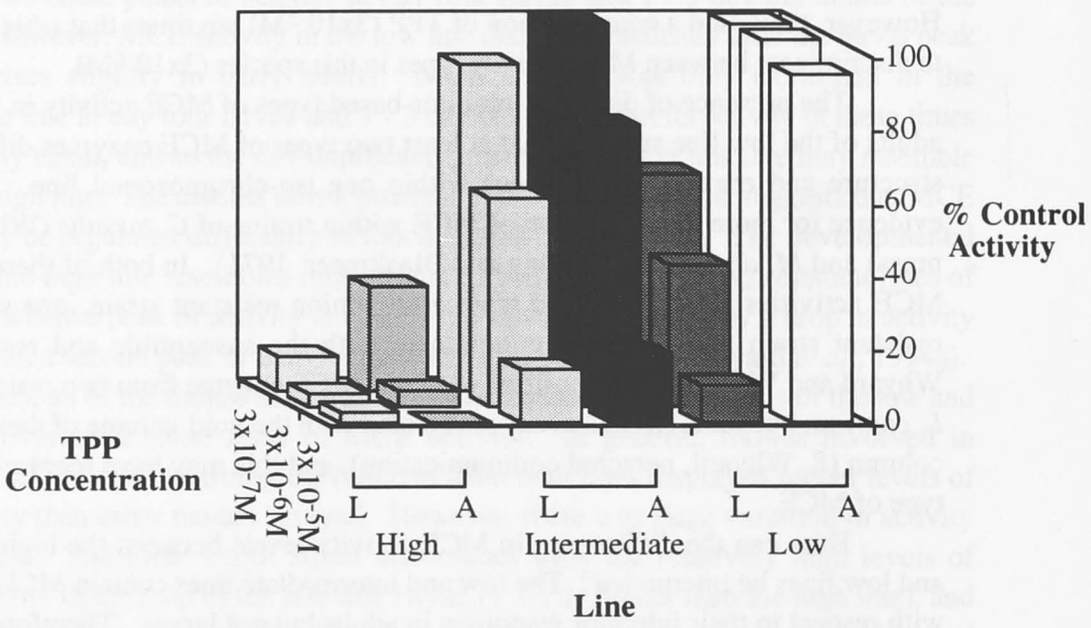


Figure 3.2 Effects of three concentrations of TPP on MCE activity in crude homogenates of day four larvae (L) and 1 - 3 day old adults (A) of the low, intermediate and high lines.

CHAPTER 4

NEGATIVE ASSOCIATION BETWEEN MALATHION AND DIAZINON RESISTANCE

4.1 Introduction

Many OPs like diazinon contain a phosphorothionate bond, which when desulphurated to a phosphate bond via mixed function oxidase activity (Eto, 1974), may be hydrolysed by a phosphatase activity (Dauterman, 1983; Section 1.3.2.3.1). Malathion contains two carboxylester bonds in addition to a phosphorodithionate linkage and may be degraded by a carboxylesterase activity without desulphuration (Eto, 1974; Section 1.4.5). A few other OPs, such as propetamphos, contain an amide bond as well as phosphorothionate and carboxylester bonds and may be degraded by either carboxylesterase, phosphatase (after desulphuration) or amidase activity (Eto, 1974; Section 1.5.3; Table 1.1).

Resistance to OPs in *L. cuprina* was first recorded in 1965 as resistance to diazinon (Shanahan, 1966; Shanahan and Hart, 1966). The frequency of diazinon resistant flies in the field was reported to be 97% in 1981 (Hughes, 1981) and it has remained high since then (98% in 1992; G. Levot personal communication). Diazinon resistance has been attributed to the acquisition of phosphatase activity as a mutant form of the E3 carboxylesterase isozyme encoded by the *Rop1* gene (Arnold and Whitten, 1976; Hughes and Devonshire, 1982; Hughes and Raftos, 1985). *Rop1* is semidominant with respect to resistance conferring 25-fold and 3.5-fold resistance on heterozygous larvae and adults, respectively, and 45- and nine-fold resistance, respectively, on homozygotes (Arnold and Whitten, 1976). E3 activity can be visualised in OP susceptible strains on native PAGE stained for esterase activity using α- and β-naphthylacetate as substrates (Hughes and Raftos, 1985; refer to Figure 2.1). However, the E3 band is not seen in OP resistant strains, presumably due to the presence of a non-staining form of the enzyme which has lost the ability to hydrolyse the artificial substrates whilst gaining the ability to hydrolyse OPs (Hughes and Raftos, 1985; Section 1.5.1).

In contrast to diazinon resistance the frequency of malathion resistant flies in the field is generally low, approximately 11% (Hughes *et al.*, 1984; G. Levot, personal communication). However, given that malathion has not been directly used to control *L. cuprina*, this level of resistance is a little surprising. Between 10- and 1000-fold resistance to malathion is associated with the presence of enhanced MCE activity and is encoded by the *Rmal* gene (Hughes *et al.*, 1984; Whyard *et al.*, 1994b; Sections 1.5.2 and 2.3). The *Rmal* and *Rop1* loci are separated by 0.7 map units ~~(mu)~~ and both form part of a cluster of several esterase genes on the left arm of chromosome 4 (Section 2.3.3).

Significant resistance to propetamphos has not yet been reported although two- to four-fold variation in tolerance does segregate in the field and propetamphos exposure in the laboratory increases both propetamphos tolerance and diazinon resistance (Levot, 1990). The mechanism of propetamphos tolerance has not been investigated and it is not known whether it involves the presumed phosphatase activity of the E3 non-staining isozyme.

The lack of a convenient assay for phosphatase degradation of diazinon type OPs has hindered biochemical analysis of the mutant E3 enzyme, but the development of a

partition assay by (Ziegler *et al.*, 1987) has permitted such analyses for MCE. A comparison of whole organism MCE specific activities across seven strains of *L. cuprina* revealed three MCE phenotypes (Section 2.3.1; Table 2.1) and the results of chapter 3 suggested that there was a structural difference between the predominant MCE forms in susceptible and resistant lines (Section 3.3.3) Specifically, the high and intermediate MCE phenotypes have 33- and 21-fold higher MCE specific activities than the low phenotype, respectively, and the high phenotype is 1000-fold more resistant to malathion than either of the other two phenotypes (Sections 2.3.1 and 2.3.2; Table 2.1). Genetic analysis mapped all three phenotypes to the *Rmal/Rop1* cluster, but complex dominance relationships were found for MCE activity and these differed from the patterns of dominance for malathion resistance (Section 2.3.3.2). Moreover, inhibitor analysis revealed three structurally different forms of MCE enzyme within and across the three specific activity phenotypes: one form was identified in low line adults, another in low line larvae plus intermediate line larvae and adults, and the third in high line larvae and adults (Section 3.3.3).

This chapter analyses the E3 and MCE polymorphisms and their relationships to diazinon and malathion resistance and propetamphos tolerance in three large samples of *L. cuprina*. Two samples are sets of fourth chromosome iso-chromosome lines, one set of 35 lines generated at random with respect to OP resistance and the other set of 20 lines screened with malathion during extraction. Results from these iso-chromosome 4 lines are then checked against a sample of five mass populations. There is an invariant association between the high MCE phenotype and malathion resistance, and a strong but not invariant association between E3 non-staining and diazinon resistance. There is also tight disequilibrium between the two resistance factors, such that malathion resistance is nearly always associated with diazinon susceptibility. Propetamphos tolerance does not appear to be associated with either MCE or E3 activities, or resistance to either malathion or diazinon.

4.2 Materials and Methods

4.2.1 Strains

The 31 new iso-chromosome 4 lines were generated in the same way as the four iso-chromosome 4 lines (RM2-6, Q4, LS2 and RM8; Section 2.2.1; Table 2.1) used in the initial survey of seven strains (Whyard *et al.*, 1994b). Briefly, wild type males were crossed with three virgin females heterozygous for a multiply inverted balancer fourth chromosome In(4)6+8+12;*Sh gl*/+ + +. Individual F_1 males heterozygous for the balancer were backcrossed to three virgin balancer strain females and the G_2 progeny (In(4)6+8+12;*Sh gl*/+ + +) were intercrossed in single pair matings. Wild type G_3 progeny from each G_2 cross were then intercrossed to establish the iso-chromosome 4 lines. These 31 new and the four original iso-chromosome lines are termed the unscreened lines because they were not screened with malathion during the extraction procedure.

The 20 other iso-chromosome 4 lines were also generated by the same procedure except that males used as parents in the G_0 and G_1 generations had previously survived a dose of 0.2% (w/v) (2µg/µl) malathion, which discriminates against malathion susceptible individuals (Hughes *et al.*, 1984). Malathion in acetone (1µl) was topically applied to the upper thorax of each parental or F_1 male, three to five days after eclosion. These 20 iso-chromosome lines are termed the screened lines.

The five mass populations used were Finley (New South Wales, collected 1991), Flinders Island (Tasmania, collected 1988), LBB (Australian Capital Territory, established before the use of OPs), Llandillo (New South Wales, collected 1989), and Murrumbateman (New South Wales, collected 1977; Foster *et al.*, 1978). All strains were maintained in the laboratory without exposure to insecticides. Refer to Figure 4.1 for collection sites for the strains from which the iso-chromosome 4 lines and mass populations were derived.

The *sv ra tg gl* marker strain used in the genetic analysis is homozygous for four recessive markers on chromosome 4: *sv* (*singed vibrissae*); *ra* (*radial vein gaps*); *tg* (*tangerine eyes*); and *gl* (*golden body*). The genetic markers have been documented by Maddern *et al.* (1986) and Weller and Foster (1993).

Adult flies were maintained on a diet of sugar and water and given a protein feed prior to oviposition. Flies were used within the first week of eclosion for all except the mapping experiments.

4.2.2 Biochemical Assays

4.2.2.1 *Malathion Carboxylesterase Assay*

MCE activity was assayed using the partition method previously described in Section 2.2.2. Individual flies were homogenised in 600µl MCE homogenisation buffer (10mM imidazole HCl, pH7.0, 1% Triton X-100(v/v), 1mM PTU and 1mM DTT) and immediately assayed for MCE activity. Aliquots of 15µl were assayed for lines expressing high or intermediate MCE activities and aliquots of 75µl were assayed for low lines and the mass populations. Specific activities were calculated as pmol malathion hydrolysed per minute per milligram of total protein and activities as pmol malathion hydrolysed per minute. Protein concentrations were determined using the method of Bradford (1976) with ovalbumin as the standard.

4.2.2.2 *Native PAGE Electrophoresis*

E3 activity was assayed using 10µl of single fly homogenate and α- and β-naphthylacetate as substrates after separation by native PAGE as previously described in Section 2.2.4, except that eserine sulphate was omitted from the staining reaction.

4.2.2.3 *Inhibitor Studies*

The MCE enzymes in adults of six screened iso-chromosome 4 lines were tested for their susceptibility to diagnostic inhibitors that distinguish low, intermediate and high MCE phenotypes (Section 3.3.3; Table 3.5). The inhibitors used were the OP paraoxon at $2x10^{-7}$M and the carboxylesterase inhibitor TPP at $3x10^{-6}$M. Aliquots of 15µl of homogenate from each line was pre-incubated with the inhibitor for 30 minutes at 25°C before assaying for MCE activity. Control assays were pre-incubated with an equal volume of inhibitor solvent (ethanol).

4.2.3 Toxicology

4.2.3.1 *LD_{50} Determinations*

Dose-mortality relationships were determined for malathion, diazinon and propetamphos for several of the iso-chromosome 4 lines. Treatment involved topical application of 1µl of OP dissolved in acetone to the upper thorax of three to five day old adults. Twenty-five flies of each sex were tested and mortality was recorded after 24 hours. Malathion was applied to the unscreened lines at seven concentrations ranging

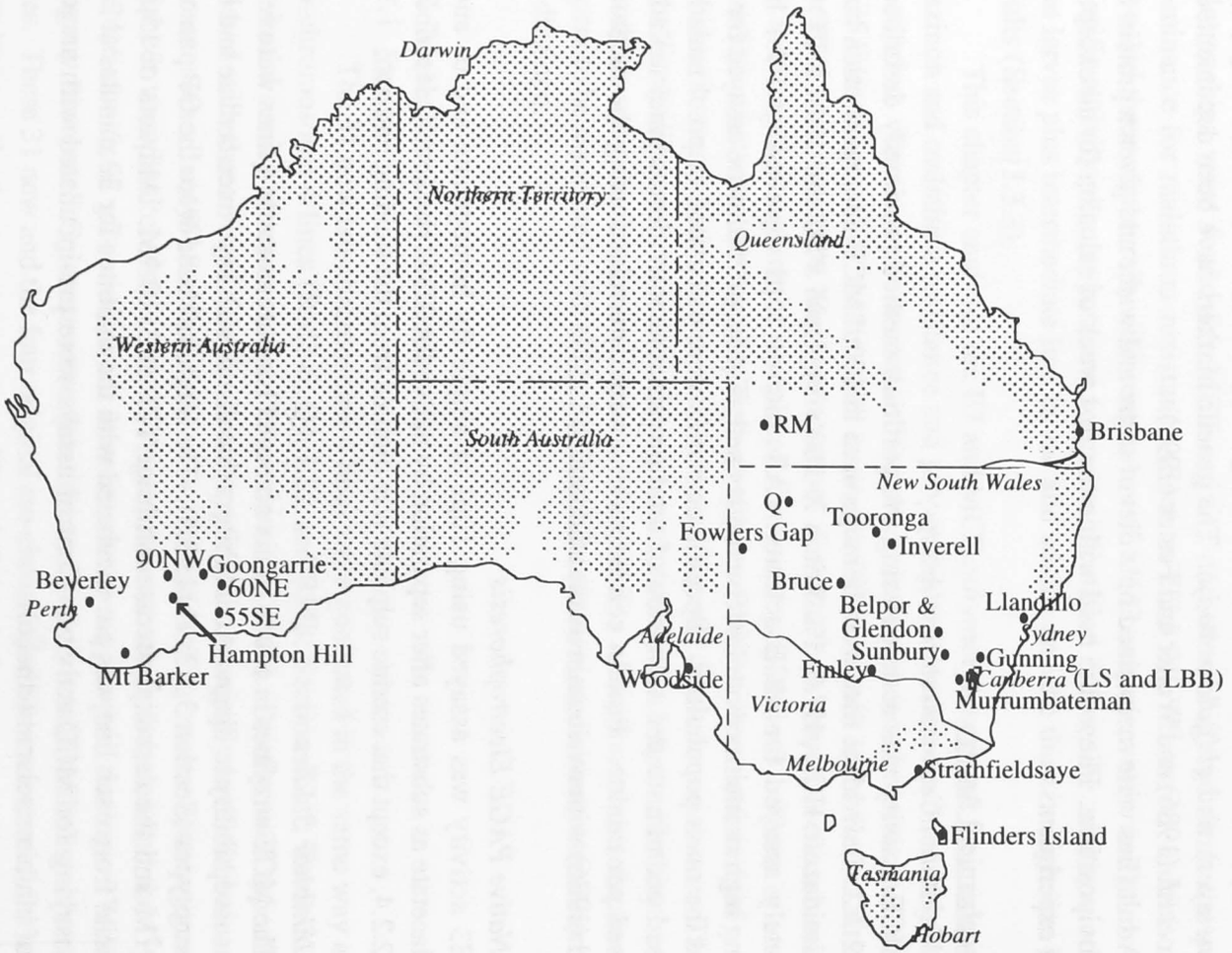

Darwin
Northern Territory
Queensland
Western Australia
South Australia
RM
Brisbane
New South Wales
Q
Tooronga
Fowlers Gap
Inverell
90NW
Goongarrie
Beverley
60NE
Bruce
Perth
55SE
Belpor &
Glendon
Llandilo
Sydney
Adelaide
Hampton Hill
Sunbury
Gunning
Finley
Canberra (LS and LBB)
Mt Barker
Woodside
Murrumbateman
Victoria
Melbourne
Strathfieldsaye
Flinders Island
Tasmania
Hobart

Figure 4.1. Map of Australia showing the collection sites of the strains from which the iso-chromosome 4 lines and mass populations were derived. Sheep growing regions are unshaded on the map (Norris, 1990).

from 0 to 0.072% (w/v) and to the screened lines at six concentrations ranging from 0 to 40% (w/v). Diazinon and propetamphos were applied to both the unscreened and screened lines at six concentrations ranging from 0 to 0.04% (w/v) for diazinon and from 0 to 0.01% (w/v) for propetamphos. Control flies were tested with acetone only. The concentration of each OP at which fifty percent of the flies died was estimated using probit analysis (Finney, 1971) as implemented in SAS, version 6.03 (SAS® Technical Report P-179, 1988).

4.2.3.2 *Discriminating Dose Assays*

Single discriminating dose assays were also carried out on 100 flies from each of the five mass populations with both malathion and diazinon. The doses used were chosen to discriminate susceptible homozygotes from heterozygote and homozygote resistant individuals. The doses were 0.2% (w/v) for malathion (2µg/µl of Hughes *et al.*, 1984) and 0.01% (w/v) for diazinon (McKenzie *et al.*, 1980), applied topically as described above (4.2.3.1). Control flies were treated with acetone and mortality was recorded after 24 hours.

4.2.4 Genetics

4.2.4.1 *Dominance of MCE Activities*

Crosses between several iso-chromosome 4 lines were set up to test the dominance relationships among the high, intermediate and low MCE specific activities. For each cross 20 F_1 individuals of each sex were assayed for MCE specific activity using 15µl per individual. The lines used and crosses established are described in Figure 4.3.

4.2.4.2 *Mapping the Malathion Resistance Locus in Woodside 5.2*

The locus conferring malathion resistance on a representative screened iso-chromosome 4 line (Woodside 5.2) was mapped with respect to visible markers on chromosome 4 using the *sv ra tg gl* maker strain. Virgin *sv ra tg gl* females were crossed with Woodside 5.2 males. Thirty F_1 progeny of each sex and 25 *sv ra tg gl* flies of each sex (as controls) were tested for resistance to 0.2% malathion as above (4.2.3.1). The F_1 females were backcrossed to *sv ra tg gl* males and the backcross progeny scored for visible markers and then screened with 0.2% malathion. Five control G_2 flies of each sex from each phenotypic class were also treated with acetone. Mortality was recorded after 24 hours.

4.3 Results

4.3.1 Unscreened Iso-chromosome 4 Lines

MCE specific activities in the 31 new unscreened lines were classified into two of the three phenotypic groups identified in the initial analysis of seven strains (Section 2.3.1 and Table 2.1; refer to Appendix 1 for the MCE specific activities for all the lines.). Twenty-eight of the lines fell into the low group and three into the intermediate group (Figure 4.2a). However, there was significant variation among the lines within each of the low and intermediate groups ($F_{30,200}$=7.45, $P \leq 0.001$ for low; $F_{3,31}$=9.0, $P \leq 0.05$ for intermediate). The range of values for the 28 new low lines (4.0±0.5 to 14.2±0.6 pmol malathion/min/mg) was comparable to that of the four original low lines (6.6±0.2 to 15.0±0.6 pmol malathion/min/mg), albeit that both the *Sh* and Dua-91 strains are not iso-

chromosome 4 lines (Section 2.3.1; Table 2.1). The range of values for the three new intermediate lines (112±5 to 168±14 pmol malathion/min/mg) was much greater than the two original intermediate strains (136±5 to 139±3 pmol malathion/min/mg), again the *sv ra gl* strain is not an iso-chromosome 4 line (Section 2.3.1; Table 2.1). ~~The new line with the highest activity (Flinders Island 5.2a; 168±14 pmol malathion/min/mg) was nearly as close to that of the high MCE line (RM8; 219±5 pmol malathion/min/mg) as it was to the overall mean of the intermediate lines (138±4 pmol malathion/min/mg).~~ Combining the data for the new lines with that of the iso-chromosome lines from the initial survey (RM2-6, Q4, LS2 and RM8 on Figure 4.2a), yielded frequencies of 86±2% for the low group, 11±2% for intermediate and 3±1% for high.

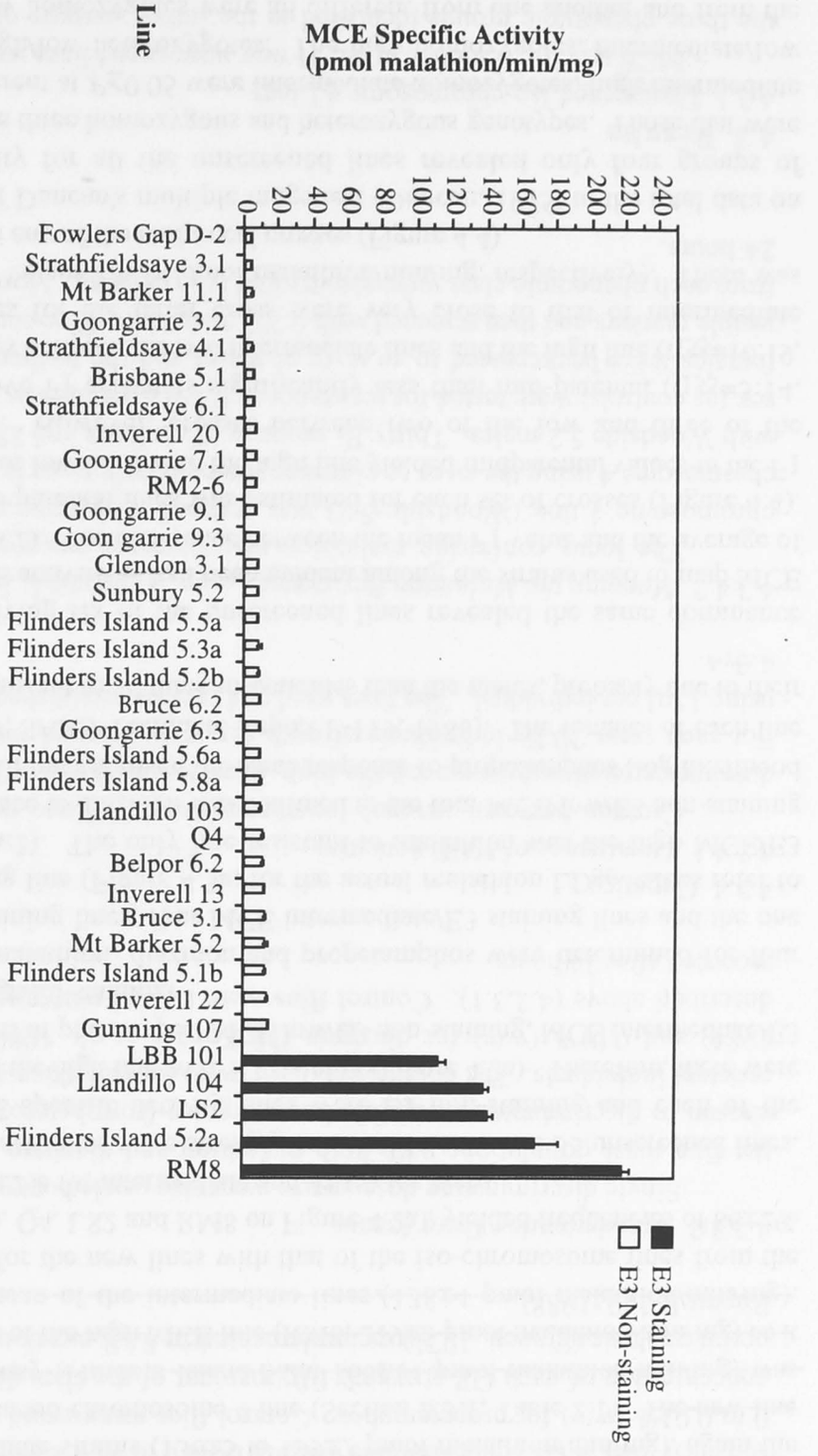
MCE and E3 activities were strongly correlated across the 35 unscreened lines. All of the low MCE specific activity lines were E3 non-staining and each of the intermediate lines and the high line were E3 staining (Figure 4.2a). Therefore, there were just three combinations of phenotypes: MCE low/E3 non-staining, MCE intermediate/E3 staining, and MCE high/E3 staining.

LD_{50}s with malathion, diazinon and propetamphos were determined for four MCE low/E3 non-staining lines, four MCE intermediate/E3 staining lines and the one MCE high/E3 staining line (Figure 4.3a; for the actual malathion LD_{50} values refer to Appendix 2, Table A.2). The only line resistant to malathion was the high MCE/E3 staining line. Resistance to diazinon was confined to the four MCE low/E3 non-staining lines (Figure 4.3a). All lines showed the same response to propetamphos (log likelihood ratio χ^2_9=4.9, P=0.84; SAS® Technical Report P-179, 1988). The females of each line were slightly more resistant to all three insecticides than the males, probably due to their larger size.

Crosses involving six of the unscreened lines revealed the same dominance relationships for MCE activity as had been evident among the strains used to map MCE activity (Section 2.3.3.2). The difference between the mean F_1 value and the average of the means for the two parental lines was estimated for each set of crosses (Figure 4.4). The crosses of the three low lines with the high line yielded midparental values in the F_1 (t_{155}=0.64, $P \geq 0.05$). However, crosses between two of the low and three of the intermediate lines gave F_1 activities significantly less than mid-parental (t_{155}=3.14, $P \leq 0.05$), as did crosses between the two intermediate lines and the high line (t_{155}=10.19, $P \leq 0.001$). F_1 values for the latter cross were very close to that of intermediate homozygotes (121±6 versus 138±4 pmol malathion/min/mg, respectively). There was no difference between any of the reciprocal crosses (Figure 4.4).

Application of Duncan's multiple range test (Duncan, 1975) to the total data on MCE specific activity for all the unscreened lines revealed only four groups of phenotypes among the three homozygous and heterozygous genotypes. Those that were not significantly different at $P \leq 0.05$ were intermediate homozygotes, high/intermediate heterozygotes and high/low heterozygotes. The high homozygotes, intermediate/low heterozygotes and low homozygotes were all different from one another and from the above group.

a.


MCE Specific Activity
(pmol malathion/min/mg)
0
20
40
60
80
100
120
140
160
180
200
220
240
Line
Fowlers Gap D-2
Strathfieldsaye 3.1
Mt Barker 11.1
Goongarrie 3.2
Strathfieldsaye 4.1
Brisbane 5.1
Strathfieldsaye 6.1
Inverell 20
Goongarrie 7.1
RM2-6
Goongarrie 9.1
Goon garrie 9.3
Glendon 3.1
Sunbury 5.2
Flinders Island 5.5a
Flinders Island 5.3a
Flinders Island 5.2b
Bruce 6.2
Goongarrie 6.3
Flinders Island 5.6a
Flinders Island 5.8a
Llandillo 103
Q4
Belpor 6.2
Inverell 13
Bruce 3.1
Mt Barker 5.2
Flinders Island 5.1b
Inverell 22
Gunning 107
LBB 101
Llandillo 104
LS2
Flinders Island 5.2a
RM8
E3 Staining
E3 Non-staining

b.

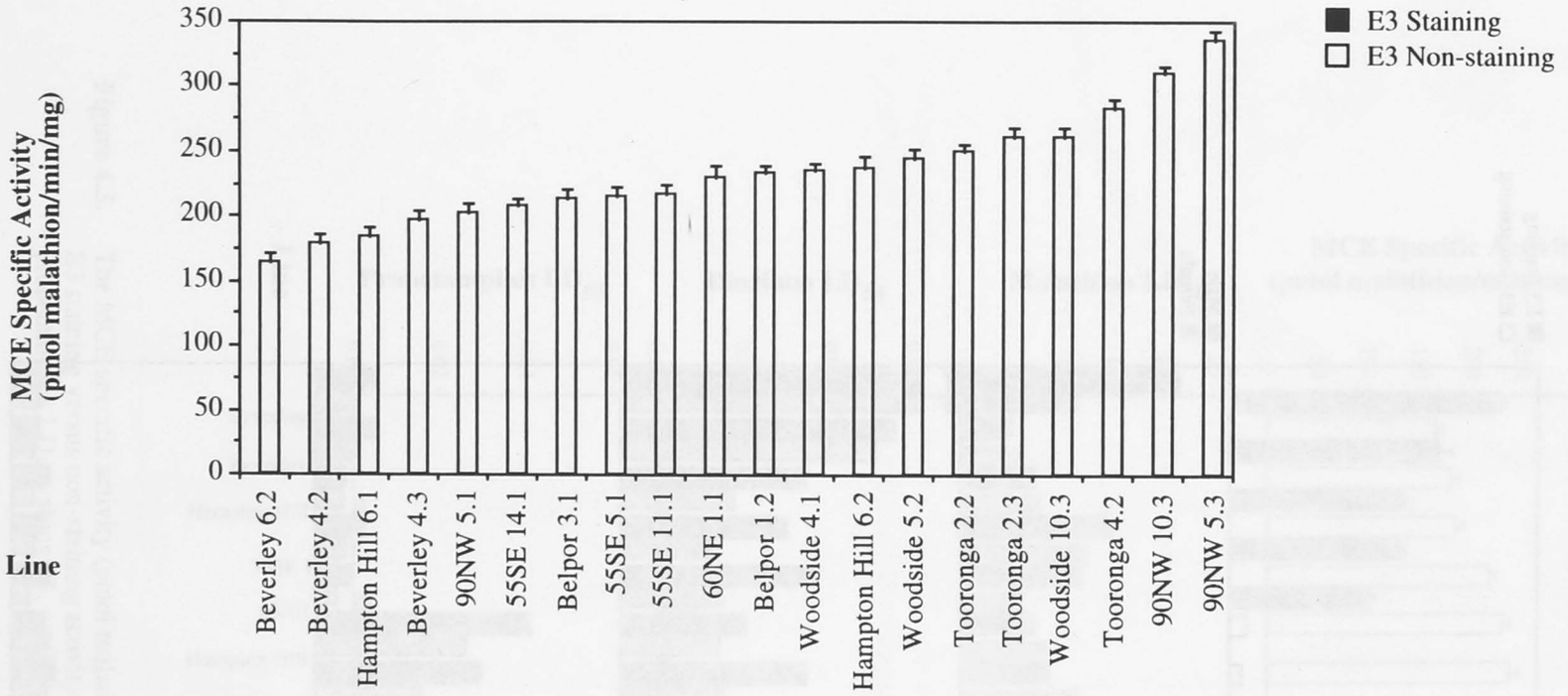
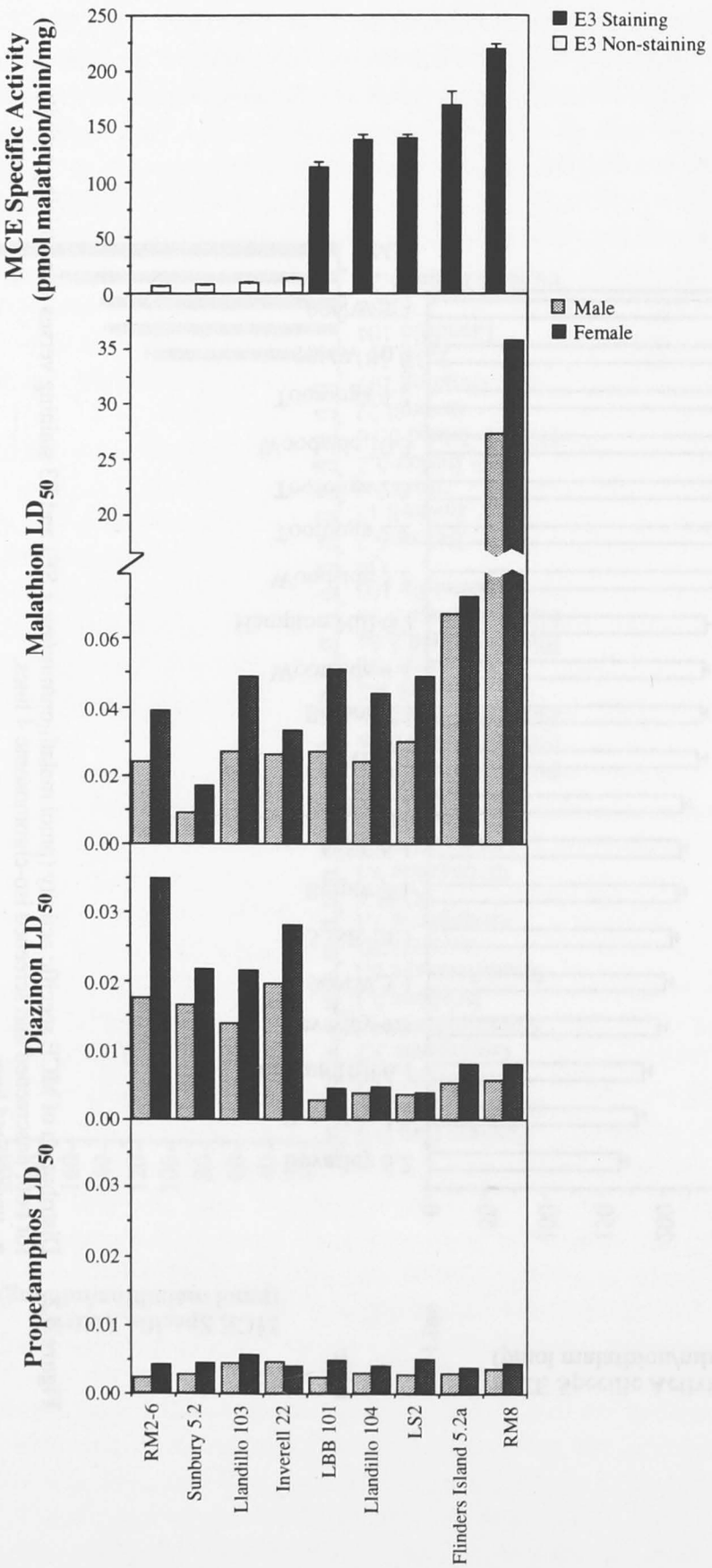


Figure 4.2. Distribution of MCE specific activity (pmol malathion/min/mg ± SE) and E3 staining versus non-staining activity for both unscreened and screened iso-chromosome 4 lines.
a. unscreened lines
b. screened lines

a.


MCE Specific Activity (pmol malathion/min/mg)
E3 Staining
E3 Non-staining
Male
Female
Malathion LD50
Diazinon LD50
Propetamphos LD50
RM2-6
Sunbury 5.2
Llandillo 103
Inverell 22
LBB 101
Llandillo 104
LS2
Flinders Island 5.2a
RM8

b.

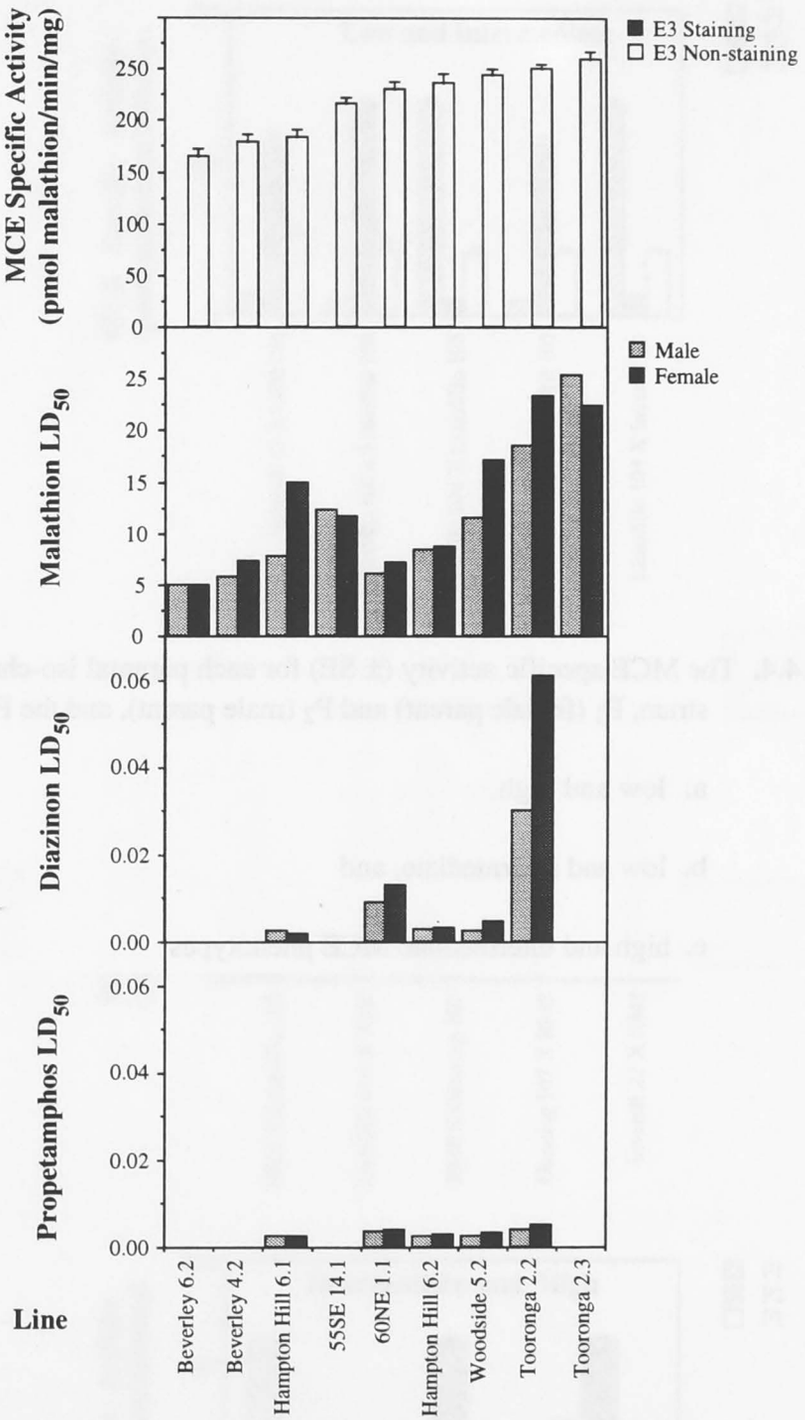


Figure 4.3. The MCE specific activity (pmol malathion/min/mg ± SE), E3 staining versus non-staining activity and malathion, diazinon and propetamphos LD_{50} values (%, w/v) for a subset of iso-chromosome 4 lines.

a. unscreened lines (Malathion LD_{50} values for RM2-6, LS2 and RM8 are taken from chapter 2; Section 2.3.2, Table 2.1).

b. screened lines (There are no diazinon and propetamphos LD_{50} values for Beverley 4.2 and 6.2, 55SE 14.1 and Tooronga 2.3).

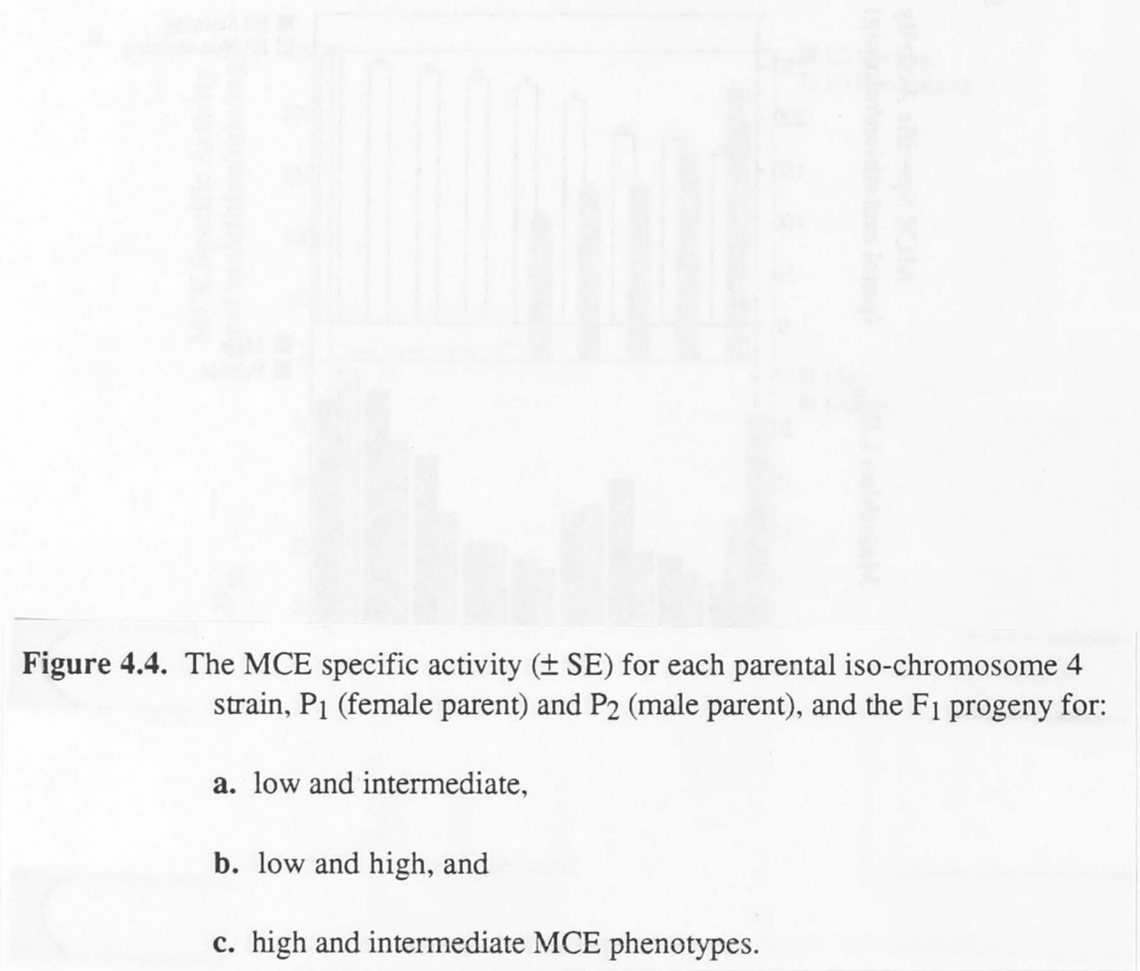


Figure 4.4. The MCE specific activity (± SE) for each parental iso-chromosome 4 strain, P_1 (female parent) and P_2 (male parent), and the F_1 progeny for:

a. low and intermediate,

b. low and high, and

c. high and intermediate MCE phenotypes.

a.
MCE Specific Activity
(pmol malathion/min/mg)
0
100
200
Inverell 22 X LBB 101
Llandillo 103 x Llandillo 104
Llandillo 104 X Llandillo 103
Llandillo 103 X LBB 101
Llandillo 104 X Inverell 22
Low and Intermediate
P1
P2
F1
b.
MCE Specific Activity
(pmol malathion/min/mg)
0
100
200
300
RM8 X Llandillo 103
Llandillo 103 X RM8
RM8 X Gunning 107
Gunning 107 X RM8
Inverell 22 X RM8
Low and High
P1
P2
F1
c.
MCE Specific Activity
(pmol malathion/min/mg)
0
100
200
300
RM8 X LBB 101
LBB 101 X RM8
Llandillo 104 X RM8
Intermediate and High
P1
P2
F1

4.3.2 Screened Iso-chromosome 4 Lines

All 20 lines generated by screening males with malathion during extraction had MCE activities in the intermediate or high range (Figure 4.2b; refer to Appendix 1 for the MCE specific activities for each of the lines). However, there was significant heterogeneity among the lines ($F_{20,197}$=25.5, $P \leq 0.001$) and no clear distinction between intermediate and high lines. Three of the lines overlapped the intermediate range of activities in unscreened lines while the remainder were at least as high or higher than the unscreened high line. Unlike the intermediate and high unscreened lines, all 20 screened lines were E3 non-staining.

Given the differences between the unscreened and screened lines, the MCE activities of six of the screened lines were tested for susceptibility to the diagnostic inhibitors TPP and paraoxon. Previous analysis of three of the original unscreened lines had shown that TPP (at $3x10^{-6}$M) does not inhibit low MCE, partially inhibits intermediate MCE and completely inhibits high MCE activities (Section 3.3.3; Figure 3.2), while $2x10^{-7}$M paraoxon partially inhibits low MCE and completely inhibits intermediate and high MCE activities (Section 3.3.3). At these concentrations both TPP and paraoxon completely inhibited the MCE activities of all six screened lines (Table 4.1), so they responded to these diagnostic inhibitors in the same way as the MCE activity of RM8, even though two of them, Beverly 6.2 and Hampton Hill 6.1, had MCE activities in the range of the intermediate MCE activity (Figure 4.2a).

Table 4.1 The effect of diagnostic inhibitors on MCE activities in crude homogenates of 1 - 3 day old adults for a subset of screened iso-chromosome 4 lines.

	Inhibitor (Concentration)	
Strain	TPP ($3x10^{-6}$M)	Paraoxon ($2x10^{-7}$M)
RM2-6 (Low)[a]	0[b]	36
LS2 (Intermediate)	23	98
RM8 (High)	96	88
Beverley 6.2	97	98
Hampton Hill 6.1	100	88
60NE 1.1	96	98
Hampton Hill 6.2	99	90
Woodside 5.2	99	88
Tooronga 2.2	97	88

[a]RM2-6, LS2 and RM8 results are included as a comparison to the unscreened iso-chromosome 4 lines (Section 3.3.3, Table 3.4)

[b]Results are the mean of three homogenates from different cultures expressed as percent inhibition of uninhibited control samples.

Nine of the screened lines were tested for their resistance to malathion and five of these were also tested for their response to diazinon and propetamphos. Each of the lines tested was resistant to malathion, with the LD_{50}s for each line significantly greater than the 0.2% used in the screening process (Figure 4.3b; the actual LD_{50} values are listed in

Appendix 2, Table A.3) There is a positive correlation between the level of resistance to malathion and MCE activity (linear regression, $F_{1,7}=6.50$, $P\leq0.01$ for females and $F_{1,7}=9.58$, $P\leq0.01$ for males), and the three screened lines that overlapped the intermediate range of MCE activities in the unscreened lines were approximately 200-fold more resistant to malathion than the unscreened intermediate lines.

Although all of the five lines tested for diazinon resistance were E3 non-staining, only one of these, Tooronga 2.2, was resistant to diazinon (Figure 4.3b). One of the other four lines, Woodside 5.2, was also tested for larval resistance and found to be susceptible (results not shown).

There was no difference among the screened lines in their propetamphos LD_{50} values (log likelihood ratio $\chi^2_6=0.24$, $P=0.99$; Figure 4.3b) nor did they differ in this respect from the unscreened lines (log likelihood ratio $\chi^2_1=0.68$, $P=0.40$). (The diazinon and propetamphos LD_{50} values are also listed in Appendix 2, Table A.3)

The gene encoding malathion resistance was mapped in a representative screened line, Woodside 5.2. All thirty male and female F_1 progeny tested survived the discriminating dose of malathion, while 286 (51.25%) of 558 G_2 progeny died. The distribution of visible markers among susceptible and resistant G_2 progeny localised the malathion resistance gene (designated *W.Rmal*) 18.5±1.6 map units from the *sv* marker and 11.5±1.4 map units from the *ra* marker, in the same region of chromosome 4 as *Rmal* (Figure 4.5). The map distances between the *sv, ra, tg* and *gl* markers were very similar to those previously reported (Weller and Foster, 1993).

4.3.3 Mass Populations

MCE activities were determined for 100 individual flies from each of five mass populations. These data cannot be directly compared with other MCE data sets because these activities are expressed on a per fly basis rather than as specific activities. Nevertheless, the distribution of activities is clearly discontinuous in each population, with one narrow peak ranging from zero to six pmol malathion/min and another, broader peak ranging from seven to 18 in the case of Llandillo and up to 34 pmol malathion/min for Finley (Figure 4.6a). Extrapolating soluble protein contents of 4mg/fly from other data sets, all activities in the lower peak should correspond to low MCE homozygotes. The frequency of these individuals is high in Finley (86%), the most recently collected population (1991), and is comparable with expectations from the frequencies of low chromosomes in the unscreened lines. However, in the other four populations, the frequencies of the putative low homozygotes are substantially lower, ranging from 9% in LBB to 42% in Murrumbateman.

Again assuming 4mg of soluble protein per fly, all individuals from each population not classified as low homozygotes have activities in the range compatible with the various intermediate and high genotypes found in the iso-chromosome lines. However, these data alone are insufficient to obtain reliable estimates for the frequencies of intermediate and high homozygotes in the mass populations, because of the evidence from the iso-chromosome lines for large and overlapping ranges of activities and complex dominance relationships in heterozygotes.

Nine or ten randomly chosen flies from each of the lower and higher peaks from each mass population were further analysed for their specific activities in order to test the validation of assigning MCE phenotypes using the 4mg of soluble protein per fly assumption (Figure 4.6b). All 49 individuals tested from the lower peak were confirmed as having MCE specific activities in the range of low homozygotes (0 to 15 pmol

malathion/min/mg). The 38 individuals originally classified as belonging to the intermediate phenotype and two as belonging to the high phenotype (assuming 4mg soluble protein per fly) from the Flinders Island, LBB, Llandillo and Murrumbateman populations had MCE specific activities that cannot be distinguished from intermediate MCE activity (ie. intermediate homozygotes, high/intermediate heterozygotes and high/low heterozygotes). One of the individuals tested from the Finley population also had an MCE specific activity resembling intermediate MCE activity (previously also resembling intermediate MCE activity assuming 4mg soluble protein per fly), whereas the MCE specific activities of other nine individuals from this population resembled those of high homozygotes (previously also resembling high MCE activity assuming 4mg soluble protein per fly). These results confirm that all the phenotypes present in the five mass populations are consistent with the genotypes of the iso-chromosome lines and the dominance patterns of these genotypes.

As was the case with the unscreened iso-chromosome lines, there was a significant non-random association between MCE and E3 activities in each of the five mass populations ($P \leq 0.001$, Fisher's exact test). All 193 individuals in the lower MCE peak were E3 non-staining, whereas all but six of the 307 individuals in the intermediate or high activity range were E3 staining, albeit that E3 staining/non-staining heterozygotes cannot be distinguished from E3 staining homozygotes. Two of the six individuals that were intermediate or high MCE and E3 non-staining came from each of the Finley, Flinders Island and Murrumbateman populations (Figure 4.6a).

The frequency of malathion resistance in the five mass populations was estimated from the frequency of individuals surviving a dose of malathion that should only kill susceptible homozygotes. This frequency varied from 4% in Flinders Island up to 27% in Finley (Table 4.2). In four mass populations malathion percent mortality was significantly higher than the estimate of the frequency of low MCE homozygotes, suggesting that there is a proportion of individuals in the higher peaks that do not carry a high MCE allele (i.e. low/intermediate MCE heterozygotes or intermediate MCE homozygotes). In contrast, the Finley population had a higher frequency of low MCE homozygotes than malathion susceptible flies (Table 4.2) suggesting that intermediate MCE activity is not common in this population.

Table 4.2 Comparison between the low MCE phenotype frequency and malathion susceptibility for the five mass populations.

Strain	Malathion Susceptibility (0.1%, w/v)	Low Phenotype Frequency	Contingency χ^2_1
Finley	73[a] ± 2.0	0.86[b]	5.2**
Flinders Island	96 ± 0.4	0.35	82.4***
LBB	92 ± 0.8	0.09	137.8***
Llandillo	88 ± 1.1	0.12	90.5***
Murrumbateman	90 ± 0.9	0.42	51.3***

[a]Results are expressed as percent mortality ± binomial standard errors.

[b]Frequency of the low MCE phenotype in each mass population.

**$P \leq 0.01$

***$P \leq 0.001$

If it is assumed that each population is in Hardy-Weinberg equilibrium, then the frequencies of the low, intermediate and high MCE genes can be estimated from the joint data on MCE activities and malathion susceptibility. The frequency of the high gene can be estimated by assuming that the frequency of malathion survivors will be high^2 + 2xhigh(1-high) (where high is the frequency of the high MCE allele), given that the malathion survivors contain both high MCE homozygotes and low/high and intermediate/high MCE heterozygotes. The frequency of the low MCE gene is the square root of the frequency of the low MCE phenotype, while the frequency of the intermediate MCE gene is calculated as the remainder in each mass population (Table 4.3).

Table 4.3 Estimates of the frequencies of the low, intermediate and high MCE genes in each of the five mass populations.

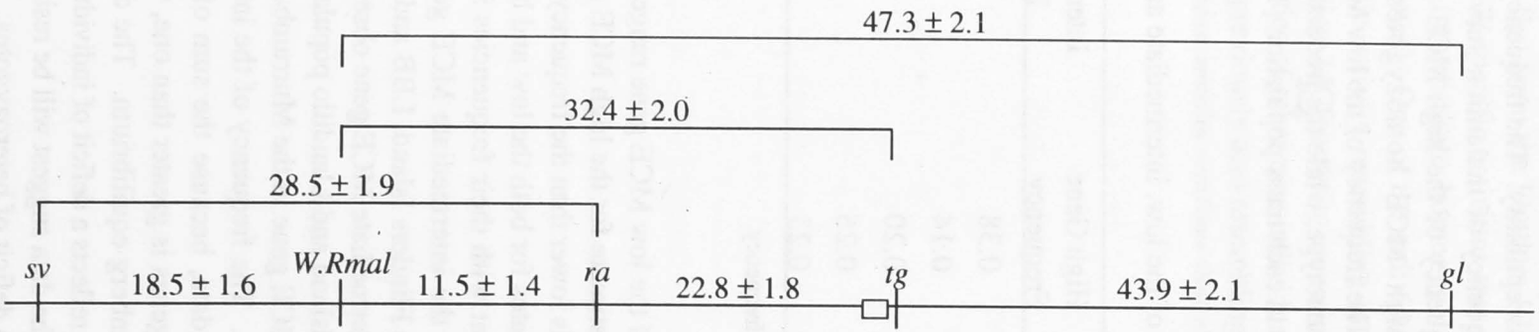
Strain	Low Gene Frequency	High Gene Frequency	Intermediate Gene Frequency
Finley	0.93	0.15	_[a]
Flinders Island	0.59	0.02	0.39
LBB	0.30	0.04	0.66
Llandillo	0.46	0.06	0.48
Murrumbateman	0.65	0.05	0.30

[a]Negative value for the intermediate MCE gene frequency.

The estimate of the frequency of the low MCE gene ranges from 0.3 in the LBB to 0.93 in the Finley population. The estimate for the high MCE gene ranges from 0.02 in Flinders Island to 0.15 in Finley and is lower than the frequency of the low MCE gene in all five mass populations. The estimates for both the low and high MCE genes in the mass populations are broadly consistent with their frequencies in the unscreened iso-chromosome lines. The frequency of the intermediate MCE gene is higher than the frequency of the high MCE gene in all the mass populations except Finley. The frequency of the intermediate MCE gene cannot be estimated from the Finley data, because the sum of the estimates of the frequencies of the low and high MCE genes is greater than one. It appears that the Finley population deviates fom Hardy-Weinberg equilibrium as there is a deficit of individuals in the intermediate MCE range and an excess of those that the data suggest will be resistant to either diazinon or malathion. This may in turn reflect a deficit of heterozygotes. It is not clear why this should be but it may be relevant to note that Finley is the most recently collected population.

The frequency of diazinon resistance in each mass population was estimated from the frequency of survivors on a concentration of diazinon that should only kill susceptible homozygotes. The estimates of diazinon resistance ranged from 84% in Finley down to 22% in LBB (Table 4.4). This frequency was significantly lower than expected from the frequency of E3 non-staining in four of the mass populations (assuming Hardy-Weinberg

a.


47.3 ± 2.1
32.4 ± 2.0
28.5 ± 1.9
sv
18.5 ± 1.6
W.Rmal
11.5 ± 1.4
ra
22.8 ± 1.8
tg
43.9 ± 2.1
gl

b.

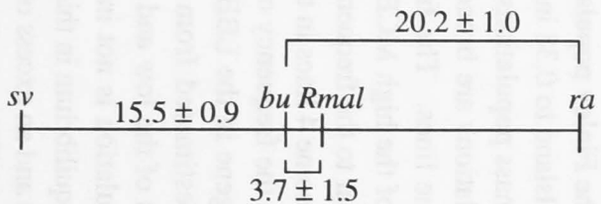

20.2 ± 1.0
sv
15.5 ± 0.9
bu Rmal
ra
3.7 ± 1.5

Figure 4.5. **a.** The linkage map of the left arm of chromosome 4 showing the results of the mapping of the locus conferring malathion resistance in Woodside 5.2 (*W.Rmal*). Numerals indicate percent crossover recovery (± SE) between the markers. The position of the centromere is indicated by □ (Foster *et al.*, 1980b).

b. The relative map position of *Rmal* (refer to Section 2.3; Figure 2.4).

proportions of heterozygotes among the E3 staining individuals, staining being dominant to non-staining). The two largest deviations from the expected frequencies are for the earliest and latest collected strains (LBB and Finley, respectively), so there is no clear correlation with the time of collection. The estimated frequency of the E3 non-staining/diazinon susceptible gene is lower than the frequency of the malathion resistance gene in all populations (Table 4.5 and refer to high gene frequency in Table 4.3). It is possible that individuals expressing this gene represent the E3 non-staining/diazinon susceptible MCE high/malathion resistant phenotypic class recovered in the screened iso-chromosome 4 lines.

Table 4.4 Comparison of percent diazinon mortality and the frequencies expected from the frequency of E3 non-staining in each of the five mass populations.

	Diazinon % Mortality		Contingency
Strain	Observed	Expected	χ^2_1
Finley	16[a] ± 1.3	0.4	624.4**
Flinders Island	19 ± 1.5	15	1.3
LBB	78 ± 1.7	49	36.7**
Llandillo	45 ± 2.5	29	12.4**
Murrumbateman	23 ± 1.8	12	10.2**

[a]Results are expressed as percent mortality ± binomial standard errors.
**$P \leq 0.01$

Table 4.5 Estimates of the frequencies of the E3 non-staining/diazinon susceptible gene in each of the five mass populations calculated as the difference between the frequencies of the E3 non-staining and diazinon resistance genes.

Strain	E3 Non-staining Genotype Frequency	E3 Non-staining Gene Frequency	Diazinon Resistance Gene Frequency	E3 Non-staining/ Diazinon Susceptible Gene Frequency
Finley	0.88	0.94	0.60	0.34
Flinders Island	0.37	0.61	0.56	0.05
LBB	0.09	0.30	0.12	0.18
Llandillo	0.21	0.46	0.33	0.13
Murrumbateman	0.44	0.66	0.52	0.14

4.4 Discussion

Only three combinations of MCE and E3 phenotypes were found among a total of 35 unscreened iso-chromosome 4 lines. Thirty-one of the lines were low MCE/E3 non-staining, four were intermediate MCE/E3 staining and another one was high MCE/E3 staining. Only high MCE/E3 staining was associated with resistance to malathion and only low MCE/E3 non-staining with resistance to diazinon. These data support other correlative studies relating high MCE activity to malathion resistance (Hughes *et al.*, 1984; Whyard *et al.*, 1994b; Section 2.3.2) and E3 non-staining to diazinon resistance (Hughes and Raftos, 1985; Parker *et al.*, 1991; McKenzie *et al.*, 1992). The data also

support prior evidence for high frequencies of diazinon resistance and low frequencies of malathion resistance in the field (Hughes *et al.*, 1984; G. Levot, personal communication).

What is novel about the unscreened iso-chromosome 4 data, however, is the negative association between MCE and E3 activities. Low MCE activity is always associated with E3 non-staining, and intermediate and high MCE activities with E3 staining. Likewise, there is a negative association between malathion and diazinon resistance such that no lines are resistant to both OPs. These associations have not been detected before because joint data for the two activities and resistance phenotypes have not been obtained previously for flies homozygous for chromosome 4. It is noteworthy that the only phenotype associated with susceptibility to both malathion and diazinon is intermediate MCE/E3 staining. The intermediate MCE/E3 staining phenotype may well be the ancestral combination, with the other two combinations being independently derived.

The three MCE activity phenotypes exhibit a complex but consistent set of dominance relationships in the F_1s of crosses involving unscreened lines, as found in the mapping experiments (Section 2.3.3.2). Crosses among several lines show no dominance between low and high, while intermediate is dominant to high and low activity is semidominant to intermediate MCE activity. These relationships differ from the patterns of dominance among the same MCE phenotypes in respect of malathion resistance. The malathion resistance associated with the high chromosome is semidominant to the malathion susceptibility associated with either the low or intermediate chromosomes (Raftos and Hughes, 1986; Sections 2.3.3 (results not shown) and 4.3.2 above). These differences in dominance bear out the activity and LD_{50} data from the original homozygous lines (Section 2.3.2) in that they show resistance is not just a simple function of high MCE specific activity.

In contrast to the distribution of MCE specific activities in the unscreened lines, the screened lines include no low MCE phenotypes. Instead they yield a continuous distribution of MCE specific activities covering a two-fold range from the upper margins of the values classified as intermediate in the unscreened lines up to values nearly 50 percent higher than the unscreened high line. Moreover, assays with diagnostic inhibitors on several of the screened lines (including two of the three in the upper intermediate range) showed that the MCE activities of the screened lines had characteristics similar to the unscreened high line and were unlike the unscreened intermediate (and low) lines. Thus the malathion screening appears to have selected for just one of the three distinct MCE phenotypes identified in the unscreened lines. However, the malathion screening did allow for variation in activity levels within this phenotype.

Importantly, the variation in activity among these lines correlates closely with differences in degrees of resistance to malathion. The variation in resistance is greater than the variation in activity (five- versus two-fold) but is still small compared to the difference between resistance and susceptibility. The results across the screened and unscreened iso-chromosome 4 lines may thus reconcile the differences between the results of chapter 3 and those of Whyard and Walker (1994). While structurally distinct MCE enzymes may lead to essentially qualitative differences in LD_{50}s between resistant and susceptible strains, quantitative differences in MCE levels within one distinct MCE phenotype may lead to quantitative differences in LD_{50}s among resistant strains. The genetic analysis of malathion resistance in Woodside 5.2 supports earlier evidence that *Rmal* encodes the resistant MCE phenotype (Raftos and Hughes, 1986). The quantitative

differences in MCE levels and resistance may be encoded by different alleles of *Rmal* or by other polymorphic loci that modify the expression of *Rmal*.

The screened lines do not show the same negative association between high (or intermediate) MCE and E3 staining activities as was evident in the unscreened lines. All the screened lines are E3 non-staining whereas the one high and all four intermediate unscreened lines are E3 staining. This difference suggests that malathion selects for a non-random combination of MCE and E3 phenotypes from amongst those that are MCE high and raises the possibility that malathion resistance may reflect some interaction between MCE and E3 activities.

The evidence for a causal connection between diazinon resistance and an E3 non-staining allele (McKenzie *et al.*, 1992) predicts that the screened lines should also be resistant to diazinon. Yet, of the five screened lines tested, four are completely susceptible to diazinon, with LD_{50}s comparable to those for E3 staining, susceptible lines from the unscreened sample. Thus the screened lines still show the negative (albeit not invariant) association between malathion and diazinon resistance that was also evident among the unscreened lines, even though they do not show the negative association between MCE and E3 activities. The screening for malathion resistance has thus resulted in isolating an allele of E3 that is not only non-staining for α-naphthylacetate, but also a null with respect to diazinon resistance. This is the first report of an E3 non-staining allele not associated with resistance.

No resistance to propetamphos was detected in any of the nine screened and five unscreened iso-chromosome 4 lines tested, nor was there any variation in tolerance either within or between these two samples. Consequently, there is also no correlation between propetamphos tolerance and diazinon resistance among the lines. These results differ from those of Levot (1990) who noted low levels of propetamphos tolerance in the field and found some correlation between this variation and diazinon resistance. The differences between these studies might be explained by another OP resistance mechanism that is not inherited on chromosome 4. Resistance to OPs mediated by mixed function oxidase activity is postulated to be encoded by *Rop2* on chromosome 6 (Arnold and Whitten, 1976; Hughes and Devonshire, 1982) and may be causally related to propetamphos tolerance.

The frequencies of individual phenotypes in the five mass populations are generally consistent with the data from the unscreened lines and prior surveys of field populations (Hughes, 1981; Hughes *et al.*, 1984). Overall, low MCE activity, malathion susceptibility and E3 non-staining are all relatively common, although the frequency distribution of MCE activities does vary substantially among populations and the frequency of diazinon resistance is surprisingly low, less than the frequency of E3 non-staining. The variation in frequency of low MCE genotypes may reflect the heterogeneity among mass populations in their collection times; low MCE activity is most frequent in the most recently collected population, Finley, and least frequent in LBB, which was collected before OPs were used in the field. The low frequency of the low MCE gene and the inferred high frequency of the intermediate MCE gene in LBB accords with the suggestion that the low MCE phenotype is not the ancestral phenotype (Section 3.4).

The anomalously low frequency of diazinon resistance does not appear to be associated with the time the mass populations were collected. All populations contain an inferred proportion of individuals that are E3 non-staining and diazinon susceptible, including LBB, which was collected before OPs were used in the field. This suggests that E3 non-staining was present in field populations before selection with diazinon. The

presence of the E3 non-staining/diazinon susceptible individuals in these mass populations also concurs with the occurrence of the diazinon susceptible E3 non-staining allele isolated in the screened iso-chromosome 4 lines. All the screened iso-chromosome 4 lines were also MCE high, and it is possible that the inferred E3 non-staining/diazinon susceptible individuals could have expressed high MCE activity, ~~as the proportion of these in each population was lower than the frequency of the high MCE gene~~. In addition to constituting the first report of an E3 non-staining/diazinon susceptible phenotype, this is also the first report of the presence of ~~the~~ an E3 non-staining phenotype in populations collected before the use of OPs.

What might be the mechanism underlying the negative cross-resistance between malathion and diazinon? One explanation could involve the history of selection with the two insecticides and the tight linkage of the two genes. The two insecticides have not been extensively used together, with diazinon mainly used on sheep and malathion used against sheep ked and sheep body louse, and in other habitats, such as market gardens (G. Levot, personal communication). Therefore, selection for diazinon resistance may have occurred on a malathion susceptible genetic background and vice versa, with the selected MCE and E3 phenotypes maintained by the relatively tight linkage between the two loci. Probably less than 400 generations of *L. cuprina* have occurred since the introduction of diazinon in 1957, so it would be reasonable to expect doubly resistant recombinants to be rare. Any selection for resistance to both OPs would appear to favour recombinants. However, fitness costs associated with resistance to each insecticide alone (McKenzie, 1993; McKenzie and O'Farrell, 1993) may be correspondingly greater for a doubly resistant chromosome, helping suppress its frequency. Such fitness costs could arise from shared, essential functions of the native MCE and E3 enzymes or from limitations to their joint expression imposed by their physical proximity or common regulatory mechanisms.

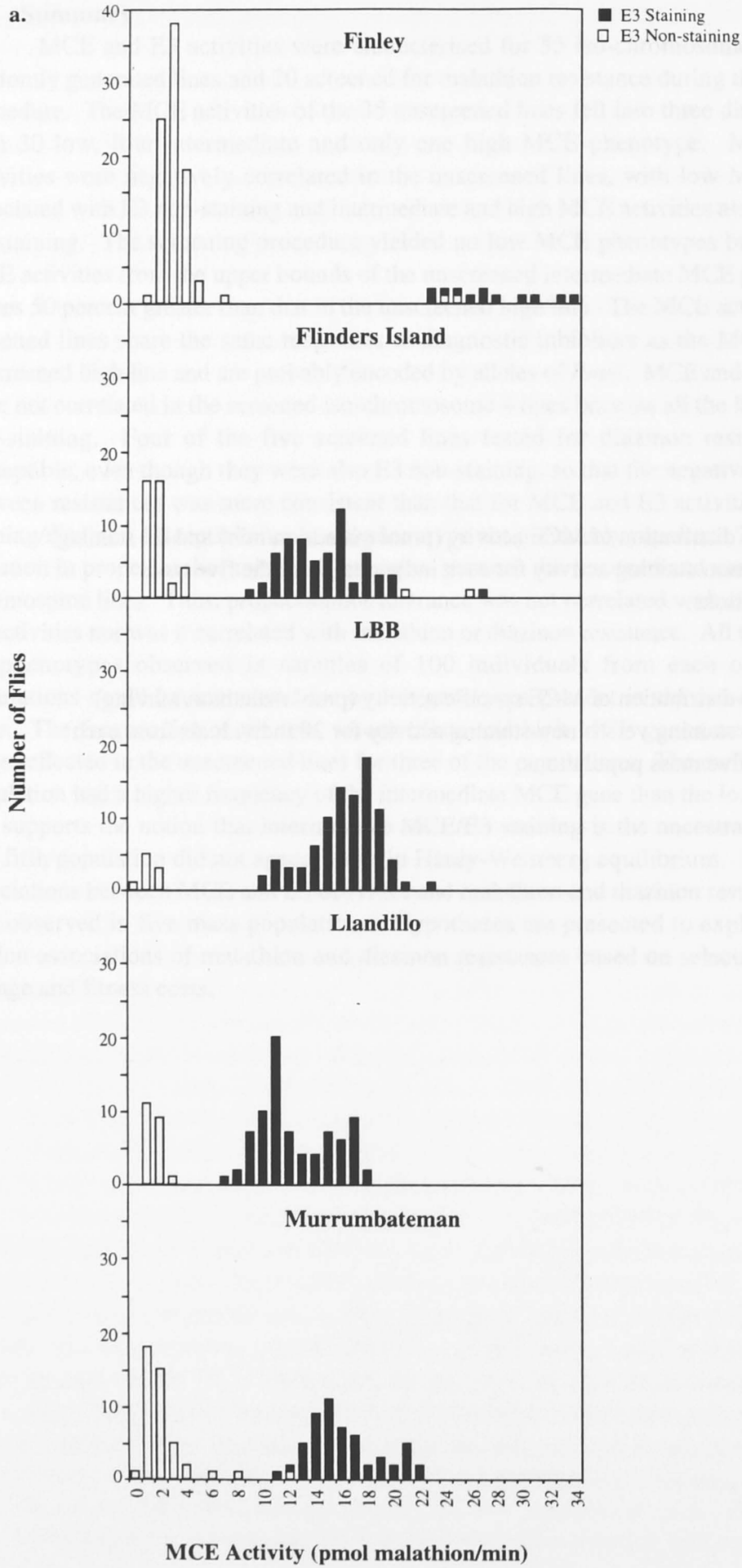
Intriguingly, there is also some evidence for a negative association between malathion and diazinon resistance in two other higher Diptera. One involves the blowfly, *Chrysomyia putoria*. Resistance to malathion in this species is conferred by increased MCE activity (Townsend and Busvine, 1969), but little is known about the resistance mechanisms to diazinon. Bervoets *et al.* (1957; reported in Busvine, 1959) claimed that the use of malathion against *C. putoria* after the development of resistance to diazinon led to the loss of some tolerance to diazinon. In the housefly, *Musca domestica*, biochemical and genetic data indicate that the mechanisms of resistance to diazinon and malathion closely resemble the MCE and E3 based mechanisms in *L. cuprina* (Oppenoorth and van Asperen, 1960; Yeoh *et al.*, 1981; Shono, 1983), albeit that other mechanisms, such as altered AChE, enhanced GST and mfo activities, also segregate in this species (Shono, 1983; Scott *et al.*, 1990b; Wang *et al.*, 1991; Devonshire and Williamson, 1993). Two early studies showed that different strains of *M. domestica* were resistant to either malathion or diazinon but not to both (Busvine, 1959; Oppenoorth, 1959). Resistance to both OPs has been subsequently reported in some other strains, but the mechanisms of this resistance is due to altered AChE, and increased GST and MCE activities without the acquistion of phosphatase activity as per E3 (Motoyama *et al.*, 1980). Thus the data for these two species and the *L. cuprina* results clearly justify further investigation into a negative association phenomenon involving carboxylesterase and phosphatase activities that might have some generality across Diptera.

4.5 Summary

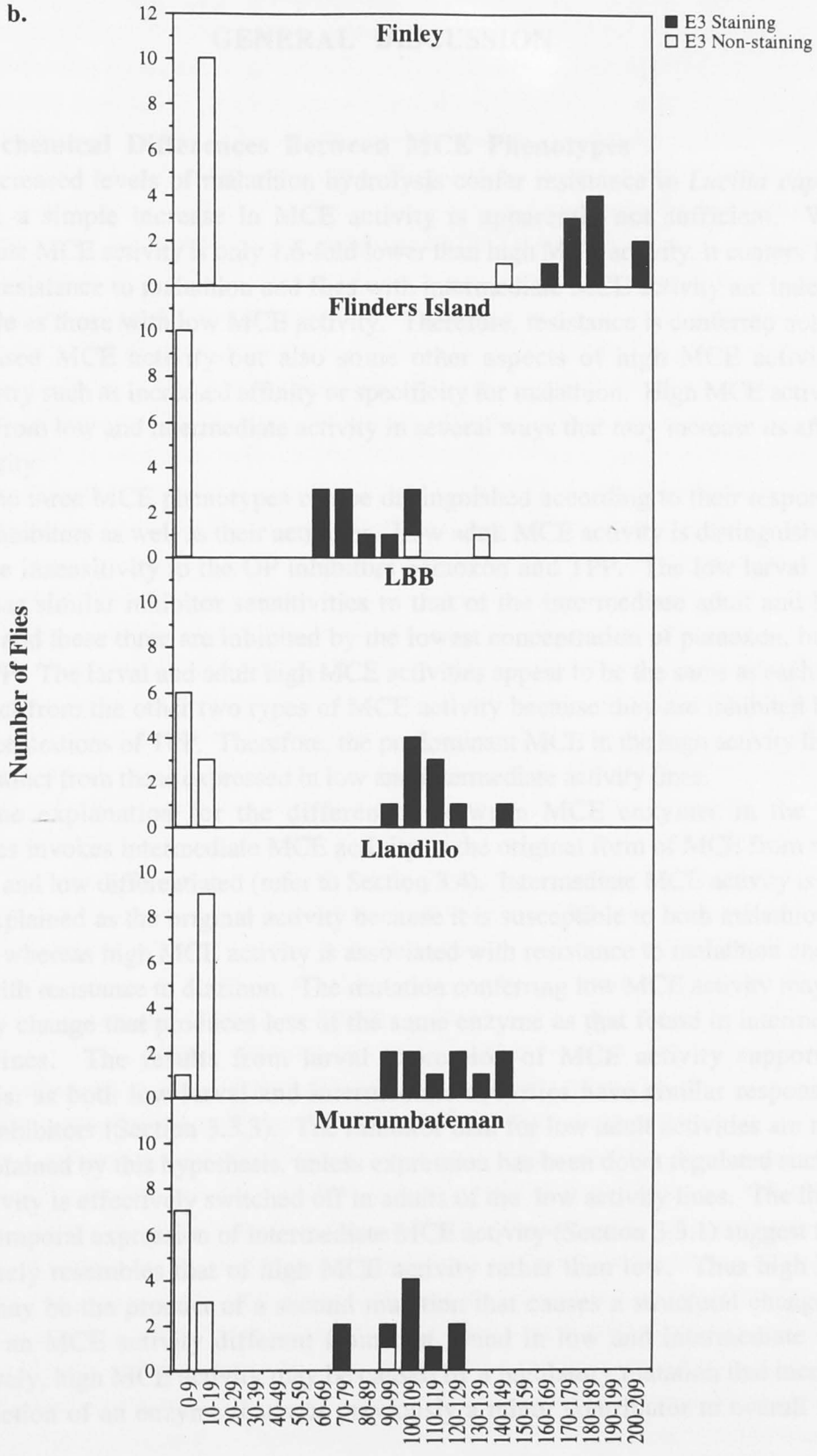
MCE and E3 activities were characterised for 55 iso-chromosome 4 lines; 35 randomly generated lines and 20 screened for malathion resistance during the extraction procedure. The MCE activities of the 35 unscreened lines fell into three distinct groups with 30 low, four intermediate and only one high MCE phenotype. MCE and E3 activities were negatively correlated in the unscreened lines, with low MCE activity associated with E3 non-staining and intermediate and high MCE activities associated with E3 staining. The screening procedure yielded no low MCE phenotypes but a range of MCE activities from the upper bounds of the unscreened intermediate MCE phenotype to values 50 percent greater than that in the unscreened high line. The MCE activities of the screened lines share the same responses to diagnostic inhibitors as the MCE from the unscreened high line and are probably encoded by alleles of *Rmal*. MCE and E3 activities were not correlated in the screened iso-chromosome 4 lines because all the lines were E3 non-staining. Four of the five screened lines tested for diazinon resistance were susceptible, even though they were also E3 non-staining, so that the negative association between resistances was more consistent than that for MCE and E3 activities. E3 non-staining/diazinon susceptible is a novel phenotype not previously reported. There was no variation in propetamphos tolerance among or between the screened and unscreened iso-chromosome lines. Thus, propetamphos tolerance was not correlated with either MCE or E3 activities nor was it correlated with malathion or diazinon resistance. All the MCE and E3 phenotypes observed in samples of 100 individuals from each of five mass populations could be accounted for by the genotypes present in the iso-chromosome lines. The frequencies of the low, intermediate and high MCE genes were similar to those reflected in the unscreened lines for three of the populations. The earliest collected population had a higher frequency of the intermediate MCE gene than the low MCE gene and supports the notion that intermediate MCE/E3 staining is the ancestral phenotype. The fifth population did not appear to be in Hardy-Weinberg equilibrium. The negative associations between MCE and E3 activities and malathion and diazinon resistances were also observed in five mass populations. Hypotheses are presented to explain the non-randon associations of malathion and diazinon resistances based on selective histories, linkage and fitness costs.

Figure 4.6. **a.** The distribution of MCE activity (pmol malathion/min) and E3 staining versus non-staining activity for each individual from the five mass populations.

b. The distribution of MCE specific activity (pmol malathion/min/mg) and E3 staining versus non-staining activity for 20 individuals from each of the five mass populations.


a.
E3 Staining
E3 Non-staining
Finley
Flinders Island
LBB
Llandillo
Murrumbateman
Number of Flies
MCE Activity (pmol malathion/min)

b.


Finley
Flinders Island
LBB
Llandillo
Murrumbateman
E3 Staining
E3 Non-staining
Number of Flies
0
2
4
6
8
10
12
0-9
10-19
20-29
30-39
40-49
50-59
60-69
70-79
80-89
90-99
100-109
110-119
120-129
130-139
140-149
150-159
160-169
170-179
180-189
190-199
200-209
MCE Specific Activity (pmol malathion/min/mg)

CHAPTER 5

GENERAL DISCUSSION

5.1 Biochemical Differences Between MCE Phenotypes

Increased levels of malathion hydrolysis confer resistance in *Lucilia cuprina*. However, a simple increase in MCE activity is apparently not sufficient. While intermediate MCE activity is only 1.6-fold lower than high MCE activity, it confers 1000-fold less resistance to malathion and flies with intermediate MCE activity are indeed as susceptible as those with low MCE activity. Therefore, resistance is conferred not only by increased MCE activity but also some other aspects of high MCE activity or biochemistry such as increased affinity or specificity for malathion. High MCE activity is different from low and intermediate activity in several ways that may increase its affinity or specificity.

The three MCE phenotypes can be distinguished according to their response to esterase inhibitors as well as their activities. Low adult MCE activity is distinguished by its relative insensitivity to the OP inhibitors paraoxon and TPP. The low larval MCE activity has similar inhibitor sensitivities to that of the intermediate adult and larval activities and these three are inhibited by the lowest concentration of paraoxon, but not that of TPP. The larval and adult high MCE activities appear to be the same as each other but distinct from the other two types of MCE activity because they are inhibited by all three concentrations of TPP. Therefore, the predominant MCE in the high activity lines is clearly distinct from those expressed in low and intermediate activity lines.

One explanation for the differences between MCE enzymes in the three phenotypes invokes intermediate MCE activity as the original form of MCE from which both high and low differentiated (refer to Section 3.4). Intermediate MCE activity is more readily explained as the original activity because it is susceptible to both malathion and diazinon, whereas high MCE activity is associated with resistance to malathion and low activity with resistance to diazinon. The mutation conferring low MCE activity may be a regulatory change that produces less of the same enzyme as that found in intermediate activity lines. The results from larval expression of MCE activity support this hypothesis, as both low larval and intermediate activities have similar responses to esterase inhibitors (Section 3.3.3). The inhibitor data for low adult activities are not so easily explained by this hypothesis, unless expression has been down regulated such that MCE activity is effectively switched off in adults of the low activity lines. The limited data for temporal expression of intermediate MCE activity (Section 3.3.1) suggest that it more closely resembles that of high MCE activity rather than low. Thus high MCE activity may be the product of a second mutation that causes a structural change and produces an MCE activity different from that found in low and intermediate lines. Alternatively, high MCE activity may be caused by a regulatory mutation that increases the production of an enzyme that was previously a minor contributor to overall MCE activity.

5.2 Genetics of MCE and E3 Activities

Some insights into the differences between high, intermediate and low MCE activities have emerged from preliminary molecular genetic data. Rapid advances have been made in the cloning and characterisation of the genes that may encode MCE activity. Four genes have been identified in *L. cuprina* that are homologous to members of the *D. melanogaster* esterase cluster (refer to Section 2.4): αE7, αE8, αE9 and αE10 (R. Newcomb, personal communication). The αE7 genes from both *D. melanogaster* and *L. cuprina*, *Dm*αE7 and *Lc*αE7 respectively, have been expressed using a baculovirus expression system. Native PAGE of the supernatants containing the expressed protein products of these genes show bands corresponding to EST23 in *D. melanogaster* and E3 in *L. cuprina* using α- and β-naphthylacetate as substrates (C. Robin and R. Newcomb personal communication). MCE assays were also performed on the expressed ~~the~~ *Lc*αE7 protein. By comparing the amount of *Lc*αE7 protein in the culture supernatant with the amount of E3 in whole fly homogenates, it was possible to calculate the MCE specific activity in the supernatant and it closely resembled that of intermediate MCE activity from crude fly homogenates (R. Newcomb and P. Campbell, personal communication). These results raise the possibility that intermediate MCE and E3 staining activities are due to the same protein.

Two important results may be explained by the same protein possessing both E3 staining and intermediate MCE activities. Firstly, intermediate MCE activity was never separated from E3 staining activity in the mapping experiments, and no intermediate MCE/E3 non-staining activity iso-chromosome 4 lines were recovered (Sections 4.3.1 and 4.3.2). Secondly, ~~statistical~~ linkage analysis ~~indicated~~ suggested that intermediate MCE activity was more likely to be an allele of *Rop1* than *Rmal* (Section 2.3.3).

Assuming that the E3 staining activity also has intermediate MCE activity, the kinetic parameters of intermediate MCE/E3 staining activity and high MCE activity can be compared. The K_M of E3 for α-naphthylacetate (30±3μM for expressed *Lc*αE7 protein; P. Campbell, personal communication and 50±16μM for the purified enzyme ~~form~~ from LS2; A. Parker, personal communication) is significantly different from that of the high MCE enzyme purified by Whyard *et al.* (1994b) from the RM strain (167±14μM) for α-naphthylacetate. Although it is clearly important to determine the kinetic constants with malathion as the substrate, the different binding affinities of these enzymes for α-naphthylacetate are sufficient to show that they are structurally distinct. Likewise a difference in kinetic properties such as K_M between the intermediate MCE/E3 staining and high MCE proteins with respect to malathion hydrolysis could account for the susceptibility of intermediate MCE flies to malathion.

While the hypothesis that E3 staining activity also has intermediate MCE activity accounts for most aspects of the total data, it requires that other aspects be reinterpreted, particularly the experiments in which MCE activity was mapped with respect to E3 activity. The only recombinant phenotype recovered from this cross was high MCE/E3 non-staining (Section 2.3.3.1). The map distance between MCE and E3 activities was thus based on five recombinants and did not take into consideration the lack of the other recombinant class: low MCE/E3 staining. This class would not be recovered if E3 staining has intermediate MCE activity because all these individuals would have the same phenotype as heterozygotes between the low MCE/E3 non-staining and high MCE/E3 staining parental haplotypes. If the E3 staining and intermediate MCE proteins are the same, the map distance between E3 and MCE activities should be revised to account for

the lack of the second recombinant class. The new map distance is two times 0.7 or 1.4±0.6 map units.

If intermediate MCE and E3 staining activity are encoded by the same gene, how many loci are there for MCE activity? It is possible that a single locus encodes all phenotypes of both MCE and E3 activities: low MCE/E3 non-staining, intermediate MCE/E3 staining and high MCE/E3 staining activities. Under this hypothesis, the protein with high MCE activity is capable of hydrolysing malathion while retaining E3 activity. Data in support of a single gene encoding all phenotypes include the purification of a single protein with MCE activity from the high MCE/E3 staining RM strain (Whyard and Walker, 1994). RM is an E3 staining strain, but no intermediate MCE activity was recovered with the purified enzyme. However, the hypothesis that an E3 staining allele also encodes high MCE activity and malathion resistance requires the rejection of a substantial body of evidence that clearly shows recombination between the two loci (Raftos and Hughes, 1986; Section 2.3.3.1).

An alternative to the single gene model proposes that there are at least two genes conferring MCE activity: the intermediate MCE/E3 gene and the low/high MCE gene. Classical genetics indicate that MCE activity is clustered within at least three esterase genes in *L. cuprina* (Section 2.3). The molecular genetic data also support at least two genes encoding MCE activity. Two of the cloned *L. cuprina* esterase genes encode products with MCE activity: the *Lc*αE10 product which does not stain with α- and β-naphthylacetate on native PAGE, and *Lc*αE7 which appears to encode E3 staining and intermediate MCE activities (R. Newcomb and P. Campbell personal communication). LcαE8 does not have any detectable MCE activity or gel phenotype and LcαE9 has not yet been expressed. Allelic alternatives at the LcαE10 or possibly LcαE9 loci may encode the high and low MCEs.

In order to identify which of the members of the *L. cuprina* cluster encodes the resistance conferring high MCE activity and whether one or more genes contribute to overall MCE activity, the locus encoding each MCE activity must be identified. This could be achieved by either expression of the cloned esterase genes or by reverse genetics. Each of the candidate cloned genes would need to be expressed from the three MCE phenotypes (low, intermediate and high), and the activities of the protein products then compared with the diagnostic inhibitors, paraoxon and TPP (Section 3.3.3). Slightly different responses to the inhibitors would be expected using the expressed protein compared with crude whole fly homogenates because the expressed protein may not be processed in the same way in the expression system and the secondary proteins present in the cell supernatants will not be the same as those in crude homogenates. However, taking all these aspects into consideration, it should still be possible to distinguish the three proteins based on their activities and inhibitor responses.

The reverse genetics approach relies on the purification of the MCE and E3 proteins and the generation of protein sequence or antibodies to these proteins to positively identify which gene or expressed protein corresponds to each biochemical phenotype. Progress on this approach has been slow due to the difficulties experienced in purifying the E3 staining and high MCE enzymes (A. Parker and S. Whyard, personal communication). Further attempts to generate larger amounts of purified high MCE enzyme are currently in progress (J. Karotam and V. Walker, personal communication). No attempts have been made to purify the low or intermediate MCE enzymes nor the E3 non-staining enzyme, since there is currently no detectable phenotype for the latter with which to follow the purification procedure.

Another approach to resolve the number of genes encoding MCE and E3 activities is to unambiguously determine the contribution of each gene product and allelic variation to resistance. One way to achieve this would be to reconstruct the resistance phenotype. If resistance is conferred by a structural mutation, transformation of different alleles at each locus into a susceptible genotype would allow the identification of the resistance conferring allele. There are several technical difficulties to this approach, particularly the lack of a transformation system for *L. cuprina*. However, the homology between the *L. cuprina* and *D. melanogaster* genes may allow the transformation of a construct carrying a structurally altered resistance allele into a susceptible *D. melanogaster* genome. On the other hand, it would be difficult to prove a causal connection for a candidate regulatory mutation by *Drosophila* transformation, because it is unlikely that the construct would be inserted in such a way as to duplicate the regulatory change necessary for resistance.

Characterisation of the kinetic parameters for the expressed proteins may help differentiate each activity and determine their contribution to overall resistance. This would provide a unique data set to relate the *in vitro* biochemical characteristics of each enzyme with its *in vivo* detoxication ability. For example, using malathion as a substrate in place of α-naphthylacetate may generate kinetic constants that differentiate low, intermediate and high MCE activities and reveal why flies with intermediate MCE activity are not resistant to malathion. It may also provide the first clear demonstration of OP hydrolysis, albeit at the carboxylester linkage, as the mechanism of resistance compared with sequestration and limited hydrolysis as exemplified by *Myzus* and *Culex* (Section 1.3.2.3.2).

5.3 Gametic Disequilibrium

Table 5.1 summarises all the MCE and E3 phenotypes recovered from the iso-chromosome 4 lines and the mass populations, except the doubly resistant line, which is considered separately below. Two combinations of MCE and E3 activities are missing: low MCE/E3 staining and intermediate MCE/E3 non-staining (albeit that three malathion resistant screened iso-chromosome lines appeared to have activities within the intermediate MCE range). The model proposing that two genes contribute to MCE activity can explain the combinations of MCE and E3 phenotypes, if it is also assumed that E3 staining and intermediate MCE activities are encoded by the same gene. Hence one gene encodes E3 staining or non-staining and intermediate MCE activities and the second encodes high or low MCE activities. Thus low MCE activity cannot also show E3 staining activity because E3 staining contributes intermediate MCE activity, which would mask the low MCE phenotype. Conversely, intermediate MCE activity cannot show E3 non-staining activity because it also has E3 staining activity (staining being dominant to non-staining).

Although the MCE and E3 phenotypes can be explained by the two gene model two aspects of the resistance phenotypes are not so easily explained. The first aspect is the E3 non-staining/diazinon susceptible phenotype. E3 non-staining has been associated with diazinon resistance in many studies and here in the unscreened iso-chromosome 4 lines and mass populations (refer to Section 4.3; Figure 4.3a), and on this basis, the high MCE/E3 non-staining lines should be resistant to both malathion and diazinon. However, with one exception, all these lines are susceptible to diazinon. (The exception, Tooronga 2.2, is resistant to both OPs, but RFLP data indicate that it carries a duplication of *Lc*αE7 and *Lc*αE8 in which one copy of each resembles those found in E3 staining

lines and the other resembles those of E3 non-staining lines; T. Boyce, personal communication).

Table 5.1 Summary of MCE and E3 activities and resistance to malathion and diazinon observed in the iso-chromosome lines.

	MCE Activity		
E3 Activity	High	Intermediate	Low
Staining	Malathion Resistant Diazinon Susceptible	Malathion Susceptible Diazinon Susceptible	
Non-staining	Malathion Resistant Diazinon Susceptible		Malathion Susceptible Diazinon Resistant

A possible explanation for the E3 non-staining diazinon susceptible phenotype is the presence of an E3 non-staining allele that has no corresponding enzyme activity (E3 null) and segregates in populations with high MCE activity. There is no direct evidence either for or against the presence of an E3 null allele. Another possible explanation is that there are interactions between the MCE and E3 enzymes that prevent the expression of double resistance. In this respect, it is also worth noting that iso-chromosome lines derived either from a parental strain resistant to malathion and diazinon by homozygosing both *Rmal* and *Rop1* loci (RopRmal 1) or from two EMS-induced diazinon resistant strains (M22.2 6.3 and M27.1 4.1) are all E3 non-staining and diazinon susceptible, as well as high MCE and malathion resistant (refer to Appendix 2, Table A.3), even though the parental lines had been selected for homozygous resistance to diazinon (McKenzie *et al.*, 1992; J. McKenzie, personal communication). In addition, another EMS induced diazinon resistant mutant, also resistant to malathion, is faintly E3 staining (F. Pike, P. Batterham and J. McKenzie unpublished data). Thus, the relationship between E3 and diazinon resistance is more complex than originally thought and the two gene model proposed above will have to be further refined before it can account for all the data.

The second problem with the two gene model, or indeed any model, is the phenomenon of the negative association between malathion and diazinon resistance in field populations of *L. cuprina*. In general terms, the negative association may be the result of an interaction of the resistance gene products with other gene products or it may reflect a regulatory mechanism that results in the expression of one or other resistance mechanisms but not both simultaneously. At this level, the results may be consistent with the "controller locus" hypothesis invoked by F. W. Plapp to explain data on *M. domestica* (Plapp, 1984). Many genes involved in insecticide resistance in *M. domestica* are located on chromosome 2, including most of the genes involved in metabolic resistance (Table 1.5). Increased expression of many of these genes has been implicated in insecticide resistance and has led to the hypothesis that a "controller locus" may be involved in regulating the expression of genes involved in metabolic detoxication (Plapp, 1984). Plapp (1984) proposed that a single, partially dominant gene on chromosome 5 is associated with multiple types of metabolic resistance. According to his model, the product of this resistance gene is a receptor protein that recognises and binds insecticides and then induces synthesis of appropriate detoxifying enzymes and he has suggested that the gene product is a high-affinity juvenile hormone binding protein that also binds xenobiotics, insecticides and plant defence chemicals (F. W. Plapp, personal

communication). Plapp's theory proposes that a mutant form of the juvenile hormone binding protein is present in resistant strains and it is able to induce the expression of different alleles of metabolic resistance genes, from those expressed in susceptible flies.

Recent work with a cytochrome P450 gene has shown that it is constitutively expressed in a cyclodiene resistant *M. domestica* strain (Andersen *et al.*, 1994). The CYP6A1 cytochrome P450 gene maps to chromosome 5, but its over-expression is controlled by a gene on chromosome 2 (Cariño *et al.*, 1994). Thus the gene is controlled in *trans* by a locus on chromosome 2. This is inconsistent with Plapp's model because the locus encoding the putative high-affinity juvenile hormone binding protein also maps to chromosome 5. Hence, if the high-affinity juvenile hormone binding protein controls the over-expression of CYP6A1, it must interact with another gene product encoded by a locus on chromosome 2. The *M. domestica* GST1 gene, *MdGST1*, is also over-expressed in insecticide resistant strains (Fournier *et al.*, 1992b) but whether its over-expression is controlled by a second gene is not known. However, the expression of GSTs in *Aedes aegypti* has been reported to be controlled by another locus or set of loci acting in *trans* (Grant and Hammock, 1992), though the gene(s) involved in regulating the expression of GST activity was not further investigated. In both *M. domestica* studies, resistance is conferred by the over-expression of the same gene product present in both resistant and susceptible strains, not the expression of a novel enzyme, and this represents a major problem with Plapp's model.

On the other hand, MCE and E3 may be co-ordinately regulated. There are many examples where the expression of genes is co-ordinately regulated. For example, the male- and female-specific doublesex products regulate sexual differentiation in *D. melanogaster* by repressing female- and male-specific terminal differentiation functions, respectively (Baker and Belote, 1983). Co-ordinate expression may explain the E3 non-staining/MCE low expression phenotype if the E3 non-staining phenotype is the result of the lack or low level of protein expression rather than a mutation that changes the enzyme's substrate specificity. Expression of E3 from *Rop1* and MCE from *Rmal* could then be down regulated or repressed resulting in low MCE/E3 non-staining activity. Switching on expression from *Rop1* and *Rmal* may result in E3 staining and high MCE activities. Intermediate MCE/E3 staining activity would then be *Rop1* on, *Rmal* off, and high MCE/E3 non-staining *Rop1* off, and *Rmal* on. However, this once again does not account for the lack of double resistance.

Another possible mechanism by which expression at the two resistance loci is influenced is by interaction with the asymmetry and fitness modifier described in *L. cuprina* (McKenzie and Purvis, 1984; McKenzie and Game, 1987; McKenzie and Clarke, 1988). The introduction of an OP resistance allele into the genome at a locus previously fixed for a susceptible allele influences biochemical and physiological processes associated with development. The level of fitness of an individual or population is often reflected by increased levels of asymmetry. The introduction of a resistance allele into *L. cuprina* is coupled with increased asymmetry and lowered fitness with respect to susceptible individuals (Whitten *et al.*, 1980; McKenzie *et al.*, 1982; McKenzie and Clarke, 1988). A dominant modifier has been selected that ameliorates the effects of the resistance allele and results in fitness and asymmetry levels similar to those of susceptible individuals (McKenzie and Game, 1987; McKenzie and Clarke, 1988).

The modifier locus (*M*) maps to chromosome 3 and is closely linked to the *white* locus (McKenzie and Purvis, 1984; McKenzie and Game, 1987). On the basis of the mapping data and the phenotypic effects of the modifier, the modifier was tentatively

identified as an allele of *Scolloped wings* (*Scl*), the *L. cuprina* homologue of *D. melanogaster Notch* (*N*). *Scl* and *N* share many phenotypic characters such as excess mechanosensory bristles, including those used to score asymmetry in *L. cuprina* (Batterham *et al.*, in press). *Scl* and *Rop1* are known to interact with respect to *Scl* phenotypes, increasing the penetrance of the *Scl* phenotype on an *Rop1* background. The modifier is also capable of influencing the *Rmal* phenotype (McKenzie and O'Farrell, 1993), implying that E3 and MCE have similar functions.

The functions of E3 and MCE in the absence of OPs are not known, nor are their sites or modes of action at the cellular level. In the absence of this information any proposition for the interaction between the resistance and *Scl* gene products is purely speculative. One theory proposed recently is that the two enzymes are involved in cell-cell interactions, based on sequence similarity between esterases in general and glutactin and neurotactin, two cell adhesion molecules (Hortsch *et al.*, 1990). It has been proposed that the *Rop1/Rmal* gene products may bind to the *Scl* protein at the cell adhesion domain just as the *N* and *Delta* gene products interact via epidermal growth factor repeats (Batterham *et al.*, in press). Therefore E3 and MCE may have similar functions in susceptible, wild type genotypes and may be involved in cell-cell interactions or cell signalling. Mutations in the *Rop1* or *Rmal* genes may then disrupt these cell-cell interactions, and the disruption could result in increased asymmetry. A subsequent mutation in the *Scl* gene that overcame the disruption caused by the resistance allele gene product would return asymmetry to "pre-mutation" levels.

Assuming that the resistance conferring mutations are structural rather than simply an increase in enzyme expression and that both E3 and MCE have a similar function or interact with the same molecule that the modifier is able to rescue, then the negative cross-resistance may be explained by the necessity for expression of at least one native enzyme for viability. E3 non-staining individuals are viable if they express the native form of MCE and vice versa. As previously mentioned the only double resistant iso-chromosome 4 line recovered was Tooronga 2.2, which appears to have two forms of E3. The gene encoding high MCE activity has not yet been identified, but it will be interesting to test whether Tooronga 2.2 also has two copies of the MCE gene, one conferring resistance and the other the native form. Identification of protein products, besides confirming the presence of one or more MCE encoding genes as described above, may also be informative on the function and possible interactions between the E3 and MCE enzymes.

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APPENDIX 1

Table A.1 Summary of the characteristics of all the iso-chromosome 4 lines generated.

Iso-chromosome 4 Line	State	Nearest Town	MCE L/I/H	E3 S/NS	MCE Specific Activity	Standard Error
Low/unscreened						
Belpor 6.2	NSW	Cowra	L	NS	10.50	0.72
Brisbane 5.1	Queensland	Brisbane	L	NS	5.10	0.26
Bruce 3.1	NSW	Trangie	L	NS	11.20	1.69
Bruce 6.2	NSW	Trangie	L	NS	8.59	0.39
FB 5.1b	Tasmania	Flinders Island	L	NS	11.74	1.63
FB 5.2b	Tasmania	Flinders Island	L	NS	8.43	0.84
FB 5.3a	Tasmania	Flinders Island	L	NS	8.04	1.87
FB 5.5a	Tasmania	Flinders Island	L	NS	7.89	0.49
FB 5.6a	Tasmania	Flinders Island	L	NS	8.83	0.05
FB 5.8a	Tasmania	Flinders Island	L	NS	8.89	1.31
Fowlers Gap D-2 (FGD2)	NSW	Broken Hill	L	NS	4.04	0.47
Glendon 3.1	NSW	Cowra	L	NS	7.58	0.74
Goongarrie 3.2	WA	Kalgoorlie	L	NS	4.40	0.12
Goongarrie 6.3	WA	Kalgoorlie	L	NS	8.82	0.46
Goongarrie 7.1	WA	Kalgoorlie	L	NS	6.30	0.13
Goongarrie 9.1	WA	Kalgoorlie	L	NS	6.73	0.22
Goongarrie 9.3	WA	Kalgoorlie	L	NS	7.49	0.29
Gunning 107	NSW	Gunning	L	NS	14.19	0.59
Inverell 13	NSW	Inverell	L	NS	11.07	0.77
Inverell 20	NSW	Inverell	L	NS	6.14	0.40

Table A.1 continued

Inverell 22	NSW	Inverell	L	NS	13.30	0.63
Llandillo 103	NSW	Penrith	L	NS	9.17	0.80
Mt Barker 5.2	WA	Albany	L	NS	11.54	3.14
Mt Barker 9.3	WA	Albany	L	NS		
Mt Barker 11.3	WA	Albany	L	NS	4.36	0.46
Q4	NSW	N. W. NSW	L	NS	9.59	0.78
RM2-6 (der-S)	Queensland	Charleville	L	NS	6.61	0.19
Strathfieldsaye 3.1	Victoria	Sale	L	NS	4.31	0.10
Strathfieldsaye 4.1	Victoria	Sale	L	NS	4.52	0.64
Strathfieldsaye 6.1	Victoria	Sale	L	NS	5.38	0.25
Sunbury 5.2	NSW	Boorowa	L	NS	7.63	0.45
<u>Intermediate/unscreened</u>						
FB 5.2a	Tasmania	Flinders Island	I	S	168.23	13.63
LBB 101	ACT	Canberra	I	S	112.17	5.26
Llandillo 104	NSW	Penrith	I	S	137.62	4.59
LS2	ACT	Canberra	I	S	139.14	3.49

Table A.1 continued

Iso-chromosome 4 Line	State	Nearest Town	MCE L/I/H	E3 S/NS	Specific Activity	Standard Error
High/unscreened						
RM8 (der-R)	Queensland	Charleville	H	S	218.97	5.01
High/screened						
55SE 5.1	WA	Kalgoorlie	H	NS	215.90	5.89
55SE 11.1	WA	Kalgoorlie	H	NS	216.89	7.82
55SE 14.1	WA	Kalgoorlie	H	NS	208.00	5.64
60NE 1.1	WA	Kalgoorlie	H	NS	229.31	9.07
90NW 5.1	WA	Kalgoorlie	H	NS	203.18	5.79
90NW 5.3	WA	Kalgoorlie	H	NS	335.19	6.86
90NW 10.3	WA	Kalgoorlie	H	NS	309.67	5.87
Belpor 1.2	NSW	Cowra	H	NS	232.71	6.02
Belpor 3.1	NSW	Cowra	H	NS	213.66	7.34
Beverly 4.2	WA	Perth	I/H	NS	179.20	7.04
Beverly 4.3	WA	Perth	H	NS	196.95	7.54
Beverly 6.2	WA	Perth	H	NS	164.78	7.20
Hampton Hill 6.1	WA	Kalgoorlie	I/H	NS	184.13	7.36
Hampton Hill 6.2	WA	Kalgoorlie	I/H	NS	236.45	9.01

Table A.1 continued

Tooronga 2.2	NSW	Warialda	H	NS	249.77	4.86
Tooronga 2.3	NSW	Warialda	H	NS	259.96	7.18
Tooronga 4.2	NSW	Warialda	H	NS	282.27	7.39
Woodside 4.1	SA	Adelaide	H	NS	234.52	6.70
Woodside 5.2	SA	Adelaide	H	NS	243.59	7.12
Woodside 10.3	SA	Adelaide	H	NS	260.92	6.50
<u>Laboratory Lines</u>						
Rop Rmal 1	Laboratory		H	NS	233.87	7.61
Rop Rmal 2	Laboratory		H	NS		
Rop Rmal 3	Laboratory		H	NS	280.50	7.53
Rop Rmal 4	Laboratory		H	NS	230.69	9.63
Rop Rmal 5	Laboratory		H	NS	258.62	6.36
M22.2 2.1	EMS-induced		H	NS	203.02	7.50
M22.2 3.1	EMS-induced			NS		
M22.2 3.2	EMS-induced		H	NS	206.20	5.33
M22.2 3.3	EMS-induced		H	NS	218.84	8.57
M22.2 6.3	EMS-induced		H	NS	263.37	9.12
M27.1 4.1	EMS-induced		H	NS	262.23	9.33

APPENDIX 2

Table A.2 Malathion, diazinon and propetamphos LD_{50} values (LD_{10}, LD_{90}) for the unscreened iso-chromosome IV strains.

	Malathion[a]			Diazinon[b]			Propetamphos[b]	
Strain	Female LD_{50}	Male LD_{50}	Resistance Status	Female LD_{50}	Male LD_{50}	Resistance Status	Female LD_{50}	Male LD_{50}
RM2-6	0.39 (0.16, 0.92)	0.24 (0.17, 0.32)	S	0.035 (0.021, 0.058)	0.018 (0.012, 0.026)	R	0.0042 (0.003, 0.007)	0.0023 (0.001, 0.007)
Inverrell 22	0.33 (0.20, 0.54)	0.26 (0.14, 0.49)	S	0.028 (0.018, 0.045)	0.020 (0.013, 0.032)	R	0.0040 (0.003, 0.006)	0.0045 (0.003, 0.007)
Llandillo 103	0.49 (0.38, 0.74)	0.27 (0.14, 0.52)	S	0.022 (0.015, 0.032)	0.014 (0.011, 0.018)	R	0.0055 (0.004, 0.008)	0.0044 (0.004, 0.005)
Sunbury 5.2a	0.17 (0.10, 0.30)	0.09 (0.05, 0.16)	S	0.022 (0.012, 0.040)	0.017 (0.010, 0.026)	R	0.0044 (0.004, 0.005)	0.0027 (0.002, 0.003)
LS2	0.49 (0.33, 0.72)	0.30 (0.21, 0.43)	S	0.004 (0.002, 0.007)	0.004 (0.003, 0.005)	S	0.0049 (0.004, 0.005)	0.0027 (0.002, 0.005)
FB 5.2a	0.72 (0.32, 1.67)	0.67 (0.14, 3.23)	S	0.008 (0.004, 0.018)	0.005 (0.004, 0.008)	S	0.0038 (0.003, 0.006)	0.0029 (0.002, 0.005)
LBB 101	0.51 (0.36, 0.73)	0.27 (0.13, 0.56)	S	0.005 (0.003, 0.006)	0.003 (0.002, 0.004)	S	0.0048 (0.004, 0.007)	0.0023 (0.001, 0.005)
Llandillo 104	0.44 (0.32, 0.60)	0.24 (0.19, 0.32)	S	0.0047 (0.0043, 0.0050)	0.004 (0.003, 0.005)	S	0.0040 (0.002, 0.008)	0.0028 (0.002, 0.003)
RM8	346 (238, 502)	261 (127, 536)	R	0.008 (0.006, 0.011)	0.006 (0.005, 0.008)	S	0.0044 (0.002, 0.008)	0.0036 (0.002, 0.007)

[a]LD values in µg/µl for both Tables A.2 and A.3

[b]LD values in % (w/v) for both Tables A.2 and A.3

Table A.3. Malathion, diazinon and propetamphos LD_{50} values (LD_{10}, LD_{90}) for the screened, EMS-induced and recombined double resistant iso-chromosome IV strains.

	Malathion[a]			Diazinon[b]			Propetamphos[b]	
Strain	Female LD_{50}	Male LD_{50}	Resistance Status	Female LD_{50}	Male LD_{50}	Resistance Status	Female LD_{50}	Male LD_{50}
Beverly 4.2	74 (52, 104)	57 (43, 76)	R					
Beverly 6.2	50 (29, 87)	49 (37, 67	R			S		
Hampton Hill 6.1	149 (119, 187)	78 (48, 127)	R	0.0017 (0.0016, 0.0019)	0.0025 (0.0023, 0.0029)	S	0.0026 (0.002, 0.003)	0.0025 (0.002, 0.003)
Hampton Hill 6.2	87 (70, 107)	85 (70, 102)	R	0.003 (0.002, 0.004)	0.0029 (0.0027, 0.0032)	S	0.0029 (0.002, 0.005)	0.0026 (0.002, 0.003)
Tooronga 2.2	233 (153, 355)	184 (101, 337)	R	0.061 (0.019, 0.199)	0.030 (0.02, 0.045)	R	0.0050 (0.003, 0.008)	0.0040 (0.002, 0.008)
Tooronga 2.3	253 (157, 407)	224 (165, 304)	R					
Woodside 5.2	170 (127, 229)	115 (75, 178)	R	0.005 (0.003, 0.007)	0.003 (0.001, 0.005)	S	0.0034 (0.003, 0.005)	0.0027 (0.002, 0.004)
55SE 14.1	117 (52, 266)	122 (90, 151)	R					
60NE 1.1	71 (39, 132)	61 (38, 98)	R	0.013 (0.007, 0.024)	0.009 (0.005, 0.016)	S	0.0039 (0.002, 0.007)	0.0038 (0.003, 0.005)
RopRmal 1	207 (166, 257)	167 (87, 319)	R	0.005 (0.004, 0.007)	0.006 0.004, 0.008)	S	0.0034 (0.002, 0.005)	0.0028 (0.002, 0.003)
M22.2 6.3	180 (148, 220)	152 (108, 215)	R	0.005 (0.004, 0.007)	0.004 (0.002, 0.006)	S	0.0038 (0.003, 0.005)	0.0030 (0.002, 0.005)
M27.1 4.1	120 (61, 239)	79 (37, 168)	R	0.004 (0.003, 0.005)	0.0028 (0.0026, 0.0031)	S	0.0030 (0.002, 0.004)	0.0030 (0.002, 0.005)